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CHARACTERIZATION OF THE AVIAN RETINOL-BINDING PROTEIN-TRANSTHYRETIN COMPLEX AND ITS ROLE IN THE OOCYTIC ENDOCYTOSIS OF RETINOL

BY

AMANDIO V. VIEIRA

A Thesis Submitted to the Faculty of Graduate Studies and Research inPartial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry and Molecular Biology.

Department of Biochemistry

Edmonton, Alberta, Canada

March 30, 1994

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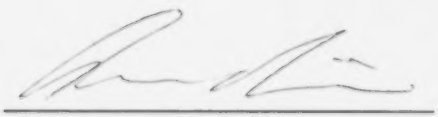

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June 9, 1994

This thesis is dedicated to
my parents, Américo and Maria
my wife, Päivi
and my son, Daniel

ABSTRACT

Retinoids are known to have essential functions in the control of cellular differentiation, particularly in the context of vertebrate embryonic development. An important and controversial aspect of retinoid research concerns the cellular uptake mechanisms for retinol (vitamin A), the precursor of active retinoids. In this study, the laying hen was used as a model system for the analysis of oocytic retinol uptake. A chicken serum retinol transport complex was purified and antibodies were raised against the two proteins of the complex, retinol-binding protein (RBP; 20 kDa) and transthyretin (TTR; 55 kDa homotetramer). Based on size exclusion and immunoaffinity chromatography, retinol fluorescence measurements, and Western blotting, evidence is presented which shows that (**1**) a ternary retinol-RBP-TTR complex exists in the chicken serum; (**2**) the same complex is present in the oocytic yolk; (**3**) the protein components of the complex, RBP and TTR, are present in clathrin-coated vesicles of the oocyte; and (**4**) the relative RBP and TTR yolk levels are similar at various stages of oocyte growth. These data support a retinol uptake mechanism which involves transfer of the retinol-RBP-TTR complex from the serum compartment into the oocyte.

A chicken RBP cDNA was cloned from a hepatic library and yielded the first known primary structure of an avian RBP. Despite high homology to mammalian RBPs, several noted features were unique to the chicken protein and its mRNA. By analysis of hepatic RNA levels, hepatic gene transcription rates, and serum protein levels, one property of chicken RBP that differentiated it from other known RBPs and most other serum-to-oocyte transporters was characterized: an absence of estrogen induction. Northern blot analysis of laying hen ovarian RNA provided the first direct evidence for lack of significant RBP synthesis by the oocyte or other ovarian cells. In the case of serum TTR, direct *in vivo* evidence for its oocytic uptake was obtained, based on the intravenous injection of labeled protein. An ultrastructural examination of the oocyte using immuno-electron microscopy revealed that RBP and TTR were incorporated into the yolk mass; their distribution was restricted to a yolk phase which is known to harbor other serum-derived yolk components and their receptors. Ligand blotting and crosslinking experiments with labeled RBP and TTR showed the presence of a binding component (~115 kDa) on ooocyte membranes for the latter, but not the former, ligand. It is suggested that this oocytic TTR receptor may mediate the uptake of retinol-RBP-TTR complex from the circulation.

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SYMBOLS AND ABBREVIATIONS

ABC	adenosine triphosphate-binding cassette
AP	adaptor protein
apo	apolipoprotein
BNHS	biotin-N-hydroxy succinimide ester
CBS	citrate buffered saline
CCV	clathrin-coated vesicle
CM	chylomicron
CPE	control of polyadenylation element
CRABP	cellular retinoic acid-binding protein
CRBP	cellular retinol-binding protein
Da	Dalton
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
ERE	estrogen response element
FA	fatty acid
HDL	high-density lipoprotein
HRP	horseradish peroxidase
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
LRAT	lecithin:retinol acyltransferase
PC	phosphatidylcholine
PBS	phosphate buffered saline
PMSF	phenylmethylsulphonyl fluoride
RA	retinoic acid
RAR	retinoic acid receptor

RBP	retinol-binding protein
RE	response element
RXR	retinoid X receptor
SA	serum albumin
TBS	tris buffered saline
TD	transmembrane domain
Tf	transferrin
TTR	transthyretin
TGF	transforming growth factor
UTR	untranslated region
u or μ	micro
VDR	vitamin D receptor
VLDL	very low density lipoprotein
VTG	vitellogenin

CHAPTER I

INTRODUCTION

Retinoids are polyisoprenoid lipids with biological activity in animal cells. Retinol (vitamin A) is the precursor of active retinoids. Specific biological functions of retinoids have been defined, particularly in the cellular processes of light detection and transcription of certain genes. It is through regulation of gene transcription that retinoids are proposed to effect their functions in embryonic development (1-4), spermatogenesis (5, 6, and refs therein), the immune system (7, 8), and inhibition of cancer cell proliferation (9, 10). Despite recent progress in delineating the mechanisms of how retinoids affect gene transcription and embryogenesis, little conclusive evidence has been obtained in studies over the past 20 years on the mechanisms of cellular retinol uptake. Retinol is proposed to be transported in the circulatory system of all vertebrates as part of a two-protein complex involving retinol-binding protein (RBP) and transthyretin (TTR). The role of the physiological RBP-TTR transport complex in mediating cellular retinol uptake has not been defined. In certain somatic cells, retinol uptake has been proposed to involve cell surface RBP receptors (Section I.3.b). RBP receptor-independent cellular uptake of retinol has also been suggested by other researchers based on retinol-RBP dissociation rates and the inability over many years to prove the existence of an RBP receptor (Section I.3.b). In consideration of the embryological roles of retinoids, it is of special importance to study how this extracellular transport complex delivers retinol to the female germ cell, the oocyte. For example, the oocytes of non-mammalian vertebrates represent a system in which the uptake, storage and metabolism of vitamins such as retinol is a necessary prerequisite for subsequent embryogenesis. The chicken oocyte, in particular, has been well characterized in terms of endocytosis of other

circulatory nutrient carriers along with their receptors; it is, thus, an excellent system in which to investigate retinol uptake and the role of the RBP-TTR complex.

A. AVIAN OOGENESIS AND EMBRYOGENESIS

The chicken is an oviparous vertebrate of the genus *Gallus*, class aves. It was established as a classic organism for the study of embryology mainly by the studies of Hamburger and Hamilton (for review see Ref. 11) and their predecessors who characterized and recorded various stages of the 21-day development of the embryo in the layed egg, i.e. after oviposition. By the time of oviposition the embryo normally has reached the early gastrulation stage of development (11).

Of the ~500, 000 oocytes present at hatching, fewer than 600 normally mature and ovulate in the lifetime of the domestic chicken and much fewer in wild birds (12). Wild birds normally lay a clutch of eggs and then stop laying in order to incubate the eggs and care for the young; but the domestic chicken (commercial hen) is a continuous breeder which can lay approximately one egg per day (~310 eggs/50 weeks) with appropriate lighting conditions and food. The chicken egg is designated as cleidoic due to its enclosure by a protective shell. Embryonic development within the cleidoic eggs of birds and reptiles presents unique problems relative to other vertebrates because all the necessary nutritional and regulatory factors must be present in the egg at the time of oviposition (with the exception of heat and rotation provided by the parent, and gas exchange with the environment).

1. Follicular Development and Morphology

At about the fourth day of development, primordial germ cells which are present at the future site of the right ovary are repositioned in the left side (future site of left ovary) and a few days later the right oviduct regresses (12). The result is that in a normal chicken only the left ovary and oviduct exist. (This regression does not occur in all bird families, however). The arrangement of the follicles in the mature ovary resembles a cluster of grapes with many visible follicles (1-35 mm diameter) in a hierarchy of different growth stages. The maintenance of this hierarchy likely involves follicular steroid hormones: Follicular estrogen production and estrogen concentration are inversely proportional to size in the most mature follicles, while that of progesterone is proportional (12). The follicles are well innervated and receive an abundant supply of blood. Each follicle contains one germ cell, the oocyte, which is surrounded by accessory somatic cell layers (Fig. I.1).

2. Oocyte Growth and Yolk Precursors

After a slow growth phase which may last up to a few years, the oocyte (and follicle) begins to grow at an increased rate ~2 months before ovulation (12). The growth rate is especially great during the last week when the mass of the oocyte increases about 150 fold! This final growth wave is believed to be mainly a result of the deposition of hepatically-synthesized blood components (yolk precursors) in the yolk of the oocyte (Fig. I.2). Little if any de novo synthesis of yolk proteins occurs in the oocyte or other follicular cells during the rapid growth phase (13, 14). Mature yolk is composed of 48% (by weight) water, 33% lipid, 17 %

protein, 1 % inorganic elements, 1 % carbohydrates and other components (15). The major lipoprotein and protein constituents are considered individually as follows:

a. Lipoproteins

Lipoproteins are structures composed of protein and lipid, mostly in a noncovalent association, which serve as mass lipid transport vehicles in the aqueous compartments of organisms. The classical lipoproteins are spherical (or discoidal in few cases) particles with a core consisting of neutral lipids (e.g., cholesteryl and retinyl esters and triglyceride) surrounded by an oriented phase of polar lipid (e.g., cholesterol and phospholipids) and the associated polypeptide(s), called apolipoproteins (Fig. I.3). The lipoprotein particles may be classified into five groups based on density: HDL, IDL, LDL, VLDL (high, intermediate, low, and very low density lipoproteins), and chylomicrons. Each group itself represents a spectrum of heterogenous particles which may differ in lipid composition and associated apolipoproteins.

Of the five lipoprotein density groups, the plasma of the laying hen contains mostly VLDL, with relatively minor levels of HDL and LDL. Laying hen VLDL is protected from lipolytic degradation in the serum by one of its apolipoproteins, apo VLDL II, which acts as an inhibitor of lipoprotein lipase (16). Hepatic synthesis of apo VLDL II is estrogen dependent (17, 18). Unlike mammals, chickens do not contain lymph-derived chylomicrons in their circulatory system; instead, dietary lipids are secreted as "protomicron" lipoprotein particles by the intestine directly into the portal vein (12). These protomicrons pass through the liver where they are removed. Unlike the chylomicrons of mammalian systems,

it is unlikely that any significant quantities of protomicrons escape liver uptake to deliver lipids to the peripheral tissues of the chicken (Sections I.C and VII.C).

Relative to laying hen plasma, that of the rooster contains low levels of VLDL. Upon estrogen treatment of roosters, however, the plasma VLDL levels increase dramatically and the plasma becomes turbid like that of laying hens. In fact, estrogen-treated roosters develop premature atherosclerosis due to the lack of an efficient VLDL removal system. Estrogen treatment of roosters or immature hens causes an immediate differentiation of hepatocytes; and new hepatocytes are recruited to the task of synthesizing yolk precursors such as VLDL (19). Estrogen increases specifically the transcription rates and mRNA half-life of the VLDL apolipoproteins, apo B (20) and apo VLDL II (17, 18), as well of a non-mammalian phospho-lipoprotein called vitellogenin, VTG (21-23). VLDL and VTG represent the major yolk precursor components of the hen plasma; both are known to be taken up specifically by the oocyte via the process of receptor-mediated endocytosis.

b. Vitamin-binding proteins

Most vertebrates possess serum proteins which transport vitamins. In chicken serum, vitamin-binding proteins have been identified for retinol (vitamin A), biotin, riboflavin (vitamin B_2), cobalamin (vitamin B_{12}), thiamine (vitamin B_1), and cholecalciferol (vitamin D_3) (24). Most of these binding proteins along with their ligands are also postulated, or have been shown, to exist in the yolk of the oocyte. Unlike the case of VLDL and VTG, however, the mechanisms of vitamin-binding protein endocytosis by the oocyte are not known. In mammalian male germ cells,

plasma membrane receptors have been postulated for cobalamin binding protein (25). In the testis, proteins that transport vitamins and other nutrients to the germ cells are likely produced by the Sertoli feeder cells which have access to the circulatory nutrient pool (5). In the case of retinol, a receptor for retinol-binding protein has been detected on cultured Sertoli cells (6).

Also unlike VLDL and VTG, the effects of estrogen on the hepatic synthesis of most of the vitamin-binding proteins are not well characterized. It is known that both in pregnant mammals and laying hens riboflavin-binding protein and thiamin-binding protein serum levels are increased as a result of the high estrogen levels (24, 26). Both of these chicken proteins are also present in the "egg white" which is secreted by the oviduct in response to estrogen.

The vitamins, or their metabolic derivatives, in the yolk are important micronutrients which have essential regulatory functions in gene expression (e.g., retinol and cholecalciferol) or as cofactors in enzyme catalysis (e.g., biotin, thiamin, riboflavin, and cobalamin). As a protein and lipid storage organelle, the yolk must be protected from oxidative damage, and some of these stored vitamins may also have additional roles as antioxidants. There are other vitamins in the yolk (e.g., niacin, ascorbic acid,) which are present at very low levels, and it is believed that the embryo can compensate for this scarcity by synthesizing its own (24).

Vitamin D is also a steroid hormone with its own nuclear receptor subfamily (Section I.C). Like other steroid hormones (e.g., cortisol, progesterone), it has a serum a carrier protein for which a specific cell surface receptor is postulated (27-29). One current area of interest in oogenesis and yolk formation research is the identification of oocyte

surface receptors for these extracellular steroid hormone-binding proteins and understanding the role of these binding proteins in the mobilization of the hormones from the yolk to the embryo during early development. Two classes of non-steroidal regulatory factors, retinoids and thyroid hormones, are also important in this respect because they have similar mechanisms of action to the steroid hormones (Section I.C) and they also possess extracellular binding proteins.

c. Other proteins in yolk

Besides being a source of amino acids, vitamins, and structural and regulatory lipids, the eggs of oviparous species must provide the embryo with minerals. One important element is iron, which is transported into the chicken egg by transferrin. Transferrin, along with riboflavin-, biotin-, and thiamin-binding proteins, also exists in the egg-white in its apo form (24). It is believed that these apoproteins provide a defense against infection by sequestering micronutrients which microbes need in order to grow. The egg of the chicken provides another form of immunity to the embryo in the form of stored IgG (30). There are also known enzymes in the yolk, although few have been well characterized. Cathepsin D is one of the best characterized; it has been shown have a role in the processing of certain protein components internalized by the oocyte, e.g. VTG, apo B (31). Unlike the cathepsin D of somatic cell lysosomes, the yolk activity does not completely degrade endocytosed proteins to amino acids and small oligopeptides; instead, a few specific fragments of the proteins are generated (31).

3. Ovulation and Completion of Egg Formation

A complex interplay between several follicular steroid hormones (e.g., progesterone produced by granulosa cells and androgens/estrogens produced by thecal cells) and hypothalamic/pituitary peptide hormones regulates follicle development and the eventual release of the oocyte from the ovary into the oviduct (ovulation) (32). This interplay is not yet completely defined, but it is believed that the progesterone and leutinizing hormone (LH) surge ~5 h before ovulation, (probably the result of a mutual positive feedback mechanism), is necessary for ovulation (12). A separate LH surge with 24 h periodicity, which occurs at the light to dark transition, is believed to be a general cue which sets the timing for ovulation and other physiological events (12). Estrogen is unlikely to be directly involved in the ovulation process, but it has important roles in the hepatocyte and oviduct cell differentiation which leads to the production of yolk precursors and egg-white components, respectively.

The normal fate of a mature follicle is to rupture and release the oocyte into the oviduct. (Sometimes, however, the follicle does not ovulate and instead follows an atretic path that ultimately leads to resorption). After release from the follicle, the oocyte begins the final meiotic maturation which is completed upon fertilization in the upper part of the oviduct (11). But regardless of whether fertilization occurs or not, the egg or oocyte, respectively, is carried through the oviduct and laid. In the oviduct, the other major events which occur are listed in the order which they occur (12): (i) deposition of albumen (egg-white) which is a gelatinous mixture composed mainly of macromolecules such as ovotransferrin, ovomucoid and other protease inhibitors, avidin, ovalbumin, lysozyme, riboflavin-binding protein; (ii) formation of the inner and outer egg

membrane sheaths; (iii) "soaking" of the albumen with large quantities (~15 g) of salts and water; (iv) calcification and subsequent pigmentation (if any) of the shell; (v) expulsion of the egg or oviposition.

B. RECEPTOR-MEDIATED ENDOCYTOSIS

Endocytosis refers to any internalization of molecules by a cell. Pinocytosis involves an invagination and vesiculization of the membrane which leads to the non-secific endocytosis of mostly fluid phase molecules. Receptor-mediated endocytosis (RME), in contrast, refers to uptake that involves specific ligand-receptor interactions at the cell surface. In RME, specific hetero-oligomeric protein complexes coat certain areas of the inner surface of the cell membrane and interact with cytoplasmic tails of transmembrane receptors (33, 34). The classic RME system involves a lattice of clathrin on the cytoplasmic surface of the membrane which surrounds complexes of adaptor proteins (APs) that contact the membrane proteins (35). The process of RME has at least two functions: (i) specific delivery of circulatory nutrient-transport proteins and their receptors into the cell, e.g. transferrin and its receptor (Tf-TfR), and (ii) attenuation of signal transduction pathways through internalization and degradation of signalling receptors and their ligands, e.g., insulin and its tyrosine kinase receptor.

1. Types of Cell Surface Receptors

Many of the different types of receptors on the cell surface can be classified based on function and number of transmembrane domains (TDs). Five major classes are noted as follows. (I) Nutrient transporters such as TfR and LDLR which have 1 TD (cf. Ref. 36) can interact with clathrin-coated membrane domains. This class of receptors does not appear to have direct roles in the transduction of mitogenic or

differentiation signals. (II) Another class with 1 TD is represented by growth factor kinase receptors (e.g., the EGF/TGF alpha receptor) which play direct roles in signal transduction that affects the cellular proliferation/differentiation state. Endocytosis of these type II receptors is ligand-induced (35, 37, 38). (III) G protein-coupled receptors with 7 TDs may be designated as another class (e.g., rhodopsin and the yeast mating factor receptors). Along with their direct roles in signal transduction, it is proposed that some are endocytosed in a ligand-dependent manner (39). (IV) Proteins with 12 TM alpha helices comprise a large group of transporters which can import or export small metabolites and drugs (40). A final group of receptors (V) with multiple TDs and, often, multiple subunits form the ligand- or voltage-gated channels (e.gs., acetylcholine receptors and the Na^+ channel) in the cell membrane. With respect to endocytosis, only a few members of the last two classes of membrane proteins (e.g., yeast STE 6) have been shown to be present in some some structures of the endocytic pathway (41).

2. Endocytic Vesicle Formation and Dynamics

Different types of cell surface pits or invaginations can pinch off and give rise to different intracellular vesicles, depending on the presence and composition of the major coat proteins. There are (i) clathrin-coated vesicles (CCVs), (ii) pinocytotic vesicles for which no cytosolic protein coat has been identified, and (iii) caveolae or plasmalemmal vesicles for which a coat protein, caveolin, has recently been identified. CCVs are considered in detail in the next section and the pinocytotic vesicles are mentioned above. In the case of caveolae, their role in endocytosis is not conclusively established. Caveolar membrane domains

are enriched in glycosylphosphatidyl-inositol(GPI)-linked proteins (33, 34, 42). Both caveolae and their GPI-proteins are proposed to mediate potocytosis, a high-affinity uptake process for low molecular weight metabolites (34, 42, 43).

a. Structure and regulation of CCVs

The first step in CCV formation is the ATP-dependent recruitment of adaptor proteins (APs) to the cytoplasmic surface of the cell membrane. AP-2, the plasma membrane protein adaptor complex, is a heterotetramer composed of alpha and beta adaptins (~100 kDa each), and a ~50 kDa and 17 kDa protein species (35). (AP-1 is used to designate AP complexes derived from intracellular membranes, and AP-3 refers to a special AP found in neurons; both APs contain other components and different isoforms of the adaptins relative to AP-2). APs are proposed to be the elements which interact with the cytoplasmic domains of cell membrane receptors (35, 38). It has not yet been conclusively established if the interaction of the APs with constitutive receptors such as TfR or LDLR results in, or is the result of, AP lattice formation. The nonconstitutive, ligand-induced tyrosine kinase receptors such as the EGF receptor (EGFR) are recruited to preformed coated pits upon ligand binding (35, 37). Research is underway to try to identify differential components for constitutive vs. nonconstitutive receptor endocytosis.

Once the AP-2 lattice has been formed with the receptor clusters, the next step is the deposition of cytosolic clathrin subunits in the form of a cage-like structure around the AP-2 complexes (35). Clathrin is composed of three heavy chains (180 kDa) and three light chains (~30

kDa). These subunits form triskelion structures which are the basic assembly unit of the clathrin cage (35).

Membrane clathrin lattices then invaginate to form the coated pit. This process requires ATP and is likely to be regulated by G proteins (35, 37, 44). Involvement of a novel GTPase, called dynamin, has been postulated for the invagination process (35, 44). Dynamin is also proposed to be involved in the subsequent step, budding, which results in a complete and separate cytosolic vesicle (33, 44).

There are many types of vesicles in the cell cytosol. The mechanisms which control the movement and fusion of exocytic, endocytic, and other intracellular trafficking vesicles are only beginning to be elucidated. For endocytic CCVs, shortly after their budding from the membrane, there is an uncoating process that removes the clathrin and AP lattices. The vesicles then fuse with a larger tubulo-vesicular organelle, the endosome (35).

The composition and function of endosomes is not yet well understood. They are dynamic structures which vary in size and activity among different cell types. Early and late endosomal compartments have been designated based on the different distribution of certain protein markers, but the functional distinctions are not all clear. An early endosomal structure or subcompartment, the sorting endosome, sorts the incoming cell membrane components into at least two groups (35): (i) integral membrane components such as the constitutive receptors (e.g., TfR) are sent back to the plasma membrane; and (ii) vesicular luminal contents along with uncoupled ligands (e.g., Tf) and many ligand-induced receptors (e.g., EGF-EGFR) are directed to lysosomes for degradation. The latter group of components are believed to move to internal vesicles within

the endosome itself, and the former group to tubular extensions of the endosome (35).

The late endosome (also called prelysosome) is defined by its lack of constitutive receptors such as TfR and the presence of some lysosomal membrane glycoproteins (35). The structures and mechanisms involved in the transition from sorting endosome to late endosome are not understood. But late endosomes can be prepared as separate entities. In the continuum of endosome structure and function, it is noteworthy that the most drastic changes in protein composition occur at the early to late transition (35). One current topic of research in this area is to try to map the routes and structures which connect the endosome, prelysosome and the trans Golgi network. For example, loading of internalized, proteolytically processed antigens onto MHC class II involves these three components (34, 35).

In order to better understand clathrin-mediated endocytosis, new components need to be identified and characterized, and many regulatory processes need to be defined. Currently, one of the known regulatory mechanisms that operates in the early endosome involves the recognition of sorting signals on the cytoplasmic domains of the constitutive receptors such as TfR and LDLR (35). These are contiguous sequences of about 4-6 amino acids which differ in the different receptors but are believed to form similar three dimensional interaction surfaces, probably a tight turn structure. A different type of sorting signal is postulated to exist for the ligand induced receptors such as EGFR (35, 37). In neither the constitutive nor ligand-induced receptors, however, is signal recognition and the sorting machinery understood.

Other important regulatory components of endocytosis and general membrane traffic are believed to be the rab family of small GTPases (34, 44-46) whose membrane association is dependent on a novel type of covalent modification: isoprenylation (Section I.C). To date, there are indications that rab 4 is involved in controlling the formation of tubulovesicular projections of the sorting endosome that mediate recycling of receptors (34, 46). Rab 5 has been found to be a minor component of CCVs and is proposed to play a role in vesicle fusion (35, 44, 46).

3. Oocytic Receptors and Endocytosis of Yolk Precursors

Oocytes from oviparous species as diverse as mosquitos and chickens have been shown to have very high endocytic activity involving CCVs (47-51). This activity is postulated to be responsible for the rapid assimilation and deposition of yolk. At present, however, little is known about the movements and transformations of CCVs in the oocytes of these species. For example, considering the large amounts of receptors involved in endocytosis and the limited biosynthetic capacity of the rapidly growing oocytes, it is likely that a ligand-receptor uncoupling organelle exists which allows regeneration of the receptor at the cell surface, but such a species is yet to be identified. Yolk vesicles or particles of different sizes, shapes, and composition have been documented, but their functional characterization is still lacking. The main storage particles in yolk (yellow yolk) are membrane-bound polygonal granules (47-53) which are believed to grow by fusion with, and pinocytosis of, smaller vesicles in the ooplasm; these smaller vesicles likely contain some of the CCV contents. As mentioned previously, true lysosomes do not exist in the rapidly growing

oocytes of the chicken (or other oviparous species). Instead, endocytosed components enter one of several storage organelles of yolk, including the polygonal granules mentioned above, which have been identified by electron microscopy (48-53). Immunoelectron microscopic analysis of chicken ovarian follicle sections with gold-conjugated antibodies has produced evidence for the differential deposition of various proteins in electron-dense and -lucent subphases of the yolk storage organelles or polygonal granules (52, 53). Similar phase structures have been identified by electron microscopic analysis after intravenous injection of gold-conjugated ligands (48, 49). Although not yet completely defined, the electron-dense structures are most often associated with components which are likely to be endocytosed by nonspecific, fluid phase mechanisms, e.g. apo AI of HDL (P. M. Vieira et al., unpublished data) and ferritin (48, 49), while the electron-lucent phase contains some components which are likely to be taken up by receptor-mediated endocytosis, e.g., VTG, VLDL (see below).

The oocytes of oviparous species contain a very high density of CCVs and clathrin-coated pits on their surfaces (47). It has been estimated that the VLDL binding capacity per unit of membrane length is at least 100-fold greater in the rapidly growing chicken oocyte than human fibroblasts or Chinese hamster ovarian cells (49). In the laying hen system, only two oocytic receptors have been characterized to date: 95 kDa and 380 kDa (54-56). Both are lipoprotein receptors structurally related to the classic 130 kDa LDL receptor of somatic cells. The 95 kDa species is likely to be directly involved in the rapid phase of oocyte growth because it is not present in a mutant chicken strain which lacks the ability to produce mature oocytes and does not ovulate (57). The mutant strain does not

accumulate the two major yolk components which are the ligands for the 95 kDa and 380 kDa receptors: VLDL (binds through its apo B domain) and VTG (binds through its lipovitellin domain) (55-57). The 95 kDa species has been detected, along with its VLDL ligand, in oocytic CCVs and in the electron-lucent phase of oocytes (53, 54).

C. RETINOIDS

The general retinoid structure (see Fig. I.4) consists of a 6-carbon ring with three additional 1-carbon branches on the ring and an 11-carbon linear branch which contains conjugated double bonds and terminates in a functional group. The three major retinoids in animal cells, retinol, retinoic acid and retinal, differ only in the oxidation state of the functional group (hydroxyl, carboxyl, or aldehyde, respectively). In addition, there are many other natural and synthetic retinoids with biological activity that have structural changes in the ring and double-bond system.

Historically, retinoids have been recognized for their roles in vision. Covalently bound retinal in the protein rhodopsin allows for light-induced conformational changes in the protein which initiate the visual process. In addition, light-induced retinal isomerization in the pineal gland (58) may be important in regulating animal circadian rythms. Another retinoid, retinoic acid, has also recently been found to be covalently linked to proteins (Ref. 59, and refs therein); the function of such retinoylated proteins, however, remains unknown. The major breakthrough in understanding additional biological functions of retinoids was provided in 1987 by the discovery of nuclear retinoid-binding proteins that can also interact with DNA (60, 61). These nuclear proteins are members of the steroid/thyroid hormone nuclear receptor family; and like the other members of this family, they can affect gene transcription in a ligand-dependent manner (62-65). This is an extensive family with over 35 members, many of which are orphan receptors (i.e., their ligands have not yet been identified). Important genes involved in cellular differentiation,

particularly in the context of embryogenesis, are regulated by retinoids and their nuclear receptors. In light of these effects, it has now become more important to understand the mechanisms by which cells, especially oocytes, can take up retinoids, and how those retinoids are mobilized and metabolized inside the cell.

Due to the hydrophobic nature of retinoids and their susceptibility to oxidative damage, a system of binding proteins exists for their transport in various cellular and extracellular compartments of animals. Besides the retinoid receptors of the cell nucleus mentioned above, there are cellular retinoid binding proteins (e.g., CRABP for retinoic acid) in the cytosol of many cells types (66, 67, and refs therein). In addition, an extracellular retinol-binding protein (RBP) is the major carrier of the precursor of active retinoids, retinol, in the circulatory system of vertebrates (68, 69). A specialized extracellular retinoid-binding protein exists in the interphotoreceptor space of the eye in animals (70, and refs therein). There is no homology in the primary structures between the members of these four groups. RBP and the cytosolic retinoid-binding proteins share some homology at the tertiary structure level (66).

1. Isoprenoid Metabolism and Isoprenylation

The ultimate origin of retinoids is the plant isoprenoid biosynthetic pathway (Fig. I.4). Acetate, derived from glycolysis and ß-oxidation of fatty acids, is the precursor of the two classical, five-carbon (C_5) isoprene units which are the building blocks of larger isoprenoids: dimethylallyl pyrophosphate (DMAP) and isopentenyl pyrophosphate (IPP). DMAP is an electrophile and IPP is a nucleophile; they are enzymatically joined to yield geranyl pyrophosphate (GPP) which is itself

an electrophile. Subsequent additions of the nucleophilic IPP C_5 unit by enzymatic transferases leads to the production of geranylgeranyl-PP (GGPP, C_{20}). In plants and other photosynthetic organisms, two GGPPs can be combined to produce phytoene which, after conjugation of the double bonds and two ring formations becomes ß-carotene.

The activity and intracellular localization of many proteins may be affected through a covalent modification involving isoprenoids. It is likely that many types of isoprenoids can be conjugated with proteins in vivo. Retinoylation (59), dolichylation (71), farnesylation (72, 73), and geranylgeranylation (72, 73) have all been reported, although only the latter two are well understood. Proteins with a cysteine close to their C-terminus (e.g., -CAAX, -CXC, -CC, where A=aliphatic and X=any amino acid) are candidates for thioether formation with geranylgeranylPP and farnesylPP (reviewed in Refs. 72, 73). In some cases, palmitoylation at a nearby cysteine (located on the N-terminal side of the isoprenylated cysteine) also occurs. Proteolytic cleavage of amino acids located on the C-terminal side of the isoprenylated cysteine (if any) also occurs followed by methyl esterification of the terminal cysteine carboxyl group (72). Isoprenylation makes the protein more hydrophobic and, as expected, can change protein-protein and protein-membrane interactions. These changes play important roles in normal cellular differentiation and proliferation (72-76), and at least one type of human ocular degenerative disease has been shown to be due to a deficiency of a geranylgeranyl transferase activity (77).

a. Carotenoids and retinoids

Carotenoids are the ultimate source of the retinoids present in animal cells. In the enterocytes (intestine), ß-carotene and other provitamin A carotenoids can be cleaved either centrally or epicentrally to yield two or one retinoid molecule, respectively (reviewed in 78). The enzymes responsible for these cleavages are not well characterized, and the mechanisms are controversial. Most of the retinoids generated are converted to retinol in the enterocytes (e.g., enzymatic reduction of retinal to retinol) (78, 79). The other major dietary source of retinoids is retinyl esters. The esters are hydrolyzed to free retinol in the intestinal lumen and the free retinol, present in mixed micelles, is absorbed by the enterocytes (78, 79).

Enterocytic retinol, bound to a cytosolic retinol-binding protein (CRBP II), is esterified (79, 80) with the sn-1 fatty acid of phosphatidylcholine by lecithin:retinol acyltransferase (LRAT) and incorporated into lipoprotein particles for delivery to the parenchymal cells of the liver (Fig. I.5). In most animals, these lipoproteins are chylomicrons (CMs). They are secreted into the lymphatic system and eventually enter the blood. The CMs and their circulatory remnants (produced by lipase activity in the liver and peripheral cells) deliver most of their core lipid content including retinyl esters to the liver; however, other tissues such as bone marrow, spleen, and adipose can take up some these CMs and remnants including their core retinyl esters (78). During the conversion of CMs into remnants, the retinyl esters are not hydrolyzed or otherwise exchanged with other lipoproteins and cellular membranes (78, 81, 82). The levels of lipids including retinyl esters in CMs are not strictly regulated, but, instead, depend on the dietary lipid load (78, 79). CMs are

usually low in the fasting state (79). In the chicken, as mentioned previously, a different system for delivery of dietary lipids to the liver exists. The intestinally derived lipoproteins (protomicrons) are delivered directly to the portal vein which carries the blood through the liver before joining the general circulation in the heart (12).

In hepatic parenchymal cells, the retinyl esters are hydrolyzed (83) and free retinol interacts with CRBP I. The retinol bound to CRBP I can have several fates, all of which involve poorly understood mechanisms: (i) transfer of the retinol to newly synthesized RBP in the endoplasmic reticulum for secretion, (ii) transfer of the retinol to neighboring hepatic stellate cells for esterification and storage in conditions of dietary retinol sufficiency, and (iii) minor levels of the retinol may be degraded or further metabolized (e.g., oxidized to retinoic acid). The hepatic stellate cells which store most of the retinyl esters in their "oil droplet" organelles (78) are key players in retinol homeostasis. The storage-vs-mobilization decisions of stellate cells are likely dependent on RBP availability (either locally expressed or transferred from parenchymal cells), CRBP I levels, and LRAT activity.

In mammals, adipocytes have also been found to store retinyl esters and secrete RBP (84). Although much less studied than hepatic tissue, the contribution made by adipocytes to total retinol mobilization and metabolism may be as much as 20% relative to liver (84). Thus, adipose tissue may prove to have a significant role in animal retinol homeostasis.

Retinoic acid (RA) is the most abundant, biologically active metabolite of retinol. Once formed, RA cannot be reduced back to retinal or retinol (85); it is either catabolized or metabolized further to other active

retinoids. The conversion of retinol to RA normally occurs in target tissues, from the retinol delivered via RBP (79, 85); in mammals, however, low levels (nM) of retinoic acid and some of its catabolites are carried bound to serum albumin (79, 85). Whether this albumin-RA has any specific roles in peripheral tissue retinoid effects is not known; it may represent only a scavenger function that brings some retinoic acid (and many other metabolites, hormones and xenobiotics) to the liver and other tissues for further degradation (86). In the case of testicular tissue, it has been shown that ^{3}H-RA-albumin complexes injected into the circulation do not contribute to the tissue RA pools (85). Also, RA-albumin, unlike the non-physiological complex RA-RBP, cannot mimic the normal retinoid-dependent increase of transglutaminase in macrophages (87). In liver and brain, however, labelled exogenous albumin-bound RA does infiltrate the normal tissue RA pools (see also Section VII).

2. Functions of Retinoids in Animal Cells

For several decades before the identification of nuclear retinoid receptors, dramatic effects of retinoids on cell proliferation and differentiation in vitro as well as on animal development and limb morphogeneis had been reported (cf. Ref. 88). With more detailed investigations, the field became also more confusing; the effects of retinoids seemed highly pleiotropic in different systems, and often there were completely different or opposite effects within the same system, when slightly different concentrations were used (reviewed in 89). For example, both retinoid deficiency and excess can lead similar malformations in mesenchyme-derived embryonic structures (89). The physiological effects of retinoids on a given cell can now be considered to

be dependent on the following factors: (i) the retinoid metabolic pathways present and their regulation (cf., 90), (ii) the amounts and isoforms of nuclear retinoid receptors and other factors that mediate the interaction of the receptors with the transcription machinery, (iii) amounts and types of steroid/thyroid nuclear hormone receptors and their ligands, (iv) presence and levels of cytosolic retinoid-binding proteins and non-protein bound retinoids (cf., (91), (v) interaction with other signal transduction pathways such as those of protein kinase C (92-95), transforming growth factor ß (89), certain oncoproteins (96, 97) and protein kinase A (98). Possibly other, poorly understood effects of retinoids in the cell which do not lead directly to changes in gene expression may also be important (89, 95, 99), e.g. protein retinoylation, general protein and lipid glycosylation, changes in membrane structure/microviscocity and gap junction com-munication. More research, however, is required to establish the physiological relevance of these effects.

a. Gene expression and embryogenesis

Apart from the covalently bound retinal in visual proteins, the nuclear retinoid-receptor proteins are the only proven direct effectors of physiological retinoid action. A nuclear retinoic acid receptor (RAR) was the first such species identified (see Fig. I.6) (60, 61). Many isoforms of RARs generated by differential ligand induction and alternative splicing (100) are now known to exist. RARs can interact both with all-trans and 9-cis retinoic acid ligands (101). More recently, a separate group of retinoid receptors, the retinoid X receptors (RXRs), has been identified (102-104); and multiple RXR isoforms have also been shown to exist (105). Unlike the RARs, only 9-cis retinoic acid can interact

with RXRs as shown by in vitro binding assays (101). RXRs can form homo- or heterodimers in vitro, but it is likely that in vivo they function preferentially as heterodimers (106). RARs can only function as heterodimers with RXRs (102-104, 106). Other related nuclear receptors for vitamin D (VDR) and thyroid hormone (TR) also function in the control of gene expression by forming heterodimers with RXRs (103, 104, 107-109). The effects of retinoids on the transcription of a particular gene are dependent on the availability of different types of retinoids, RXRs, RARs as well as VDRs and TRs along with their respective ligands. For example, the interrelation between TRs and RARs and their respective ligands is documented (110, and refs therein). In addition, the DNA response elements that interact with the nuclear receptors have a selectivity for a particular heterodimer as determined by the spacing between the two short DNA core sequence elements which interact with each protein of the heterodimer (Fig. I.6).

The large family of nuclear steroid/thyroid/retinoid receptors can be divided into two groups based on the organization of the DNA response elements and the association (if any) with cytosolic heat shock proteins (hsp): (i) steroid receptors, with the exception of the vitamin D receptor, (ii) VDR/retinoid/thyroid/orphan receptors. The steroid hormone receptors (88, 109, 111), but not the others, interact with a complex of hsp 90, hsp 70 and other proteins in the cell cytosol (and perhaps also in the nucleus). The binding of the steroid hormone leads to the dissociation of the complex and formation of receptor dimers which can interact with the transcription machinery. The steroid hormone receptors interact with inverted repeats of the DNA core binding elements with a 3 bp spacing between the elements; the other receptors interact

preferentially as heterodimers of RXR with core DNA elements in direct repeat configurations as shown in Fig. I.6.

Retinoids are important for normal embryogenesis in many organisms, including the chicken (4, 89, 112-114). In Xenopus, for example, concentration gradients of biologically active retinoids exist and are likely to be involved in posterior-anterior axis formation and neurulation (115). In chickens and other vertebrates, retinoic acid has been shown to induce the expression of specific homeobox genes whose products are involved in establishing normal embryo morphology (112 and refs therein). In addition an important morphological control centre in the embryo, Hensen's node, may be an important site of RA synthesis in the chicken (116). It is known that embryos from chickens raised on a retinol-deficient diet will die before the second day of incubation due mainly to cardiovascular structural abnormalities (113, and refs therein). Presumably, in the chicken and other egg-laying animals, serum retinol must be taken up by the rapidly growing oocytes, and is then stored, metabolized, and eventually performs its functions at specific stages of embryogenesis. Recent results from the initial studies of retinoids and their nuclear receptors on avian (quail) development suggest that all-trans retinoic acid, generated from stored retinol, along with the RARß subtype and RXR have important early (~30 hr post oviposition) functions in the embryo (113). Surprisingly, the 9-cis retinoic acid isomer does not appear to be necessary for these early functions (113).

3. Transport and Cellular Uptake of Retinol

The effects of retinoids in embryonic development and tumorigenesis are also dependent on the levels of the cellular retinol- and

retinoic acid-binding proteins (CRBPs, CRABPs) because they control the availability, and perhaps delivery, of retinoids to the nuclear receptors (67, 91). In some somatic cells, CRBPs, may also play a direct role in the uptake of retinol from the circulation by acting as cytosolic acceptors of retinol from the inner leaflet of the plasma membrane or from hypothetical retinol membrane transporters (discussed in Section VII.A), perhaps like those which may transport fatty acid (67) or dolichol (117). This uptake-related function of CRBPs is controversial as are the mechanisms of retinol uptake in general. In this section, the extracellular retinol-protein transport complex (R-RBP-TTR) as well as its role in retinol delivery will be reviewed. The results discussed were derived mostly from mammalian studies.

a. Serum retinol-binding protein and transthyretin

The 20 kDa RBP protein is a member of the lipocalin family of proteins which are proposed, or have been shown, to be involved in the transport of small hydrophobic molecules (see Fig. I.7). The lipocalins for which the 3-dimensional crystal structure has been resolved are known to form a ß-barrel structure composed of two antiparallel ß-sheets which sequesters small lipophilic molecules such as retinol (118-121). The RBP present in serum is synthesized mainly in the liver (69 and Chapter I); although, small amounts of RBP mRNA have also been found in ocular and adipose tissues, and, at even lower levels, other internal organs (84, and refs therein). RBP contains no known post-translational modifications (69, 122, 123). Hepatic secretion of RBP is dependent on the availability of retinol (69, 124). Although, RBP can also bind RA, only

retinol binding leads to a holo-RBP that is competent for export from the endoplasmic reticulum (122).

In the circulation, RBP is known to be associated in a 1:1 complex with the homotetrameric, 55 kDa TTR protein. (69, 125) Most of the TTR present in the blood is also synthesized by the liver (69). It is not known if the physiologic holoRBP-TTR complex forms in the hepatocytes during holo-RBP and TTR secretion or in the blood after secretion.

b. Cellular uptake of retinol, RBP, and TTR

Reported retinol uptake rates in epithelial cells of the cornea and intestine (126), and liver parenchymal cells (127) are similar to or lower (5X lower in the latter case) than the rate of dissociation of retinol from RBP, measured in an in vitro, PC vesicle-dependent system (126). This implies that the retinol can dissociate from RBP sufficiently fast to account for its uptake into cells; and it is compatible with an uptake mechanism where retinol dissociates from RBP, partitions into the cell membrane, and is then sequestered internally by a CRBP. In such a scheme, no membrane receptor for holo-RBP would be required. Indeed, several studies in different cell types (see 127 and Refs therein) have provided evidence that an RBP receptor is neither present nor necessary. Although the question of retinol uptake is still controversial, there are several observations and arguments which suggest that this spontaneous retinol uptake may not be the most important mechanism in many cells: (i) The dissociation rate for retinol from RBP calculated in the above in vitro system may not reflect the in vivo rate. (ii) The dissociation rate of retinol from the physiological RBP-TTR complex may be much lower than from RBP alone. (iii) As mentioned by Senoo et. al. (128), white blood cells,

which do not store retinyl esters, contain ~1000X more retinol than the longer-lived red blood cells. The unregulated dissociation of retinol proposed above is unlikely to account for this difference because both cell types are exposed to the same retinol concentrations. One may argue that the presence of higher levels of cellular retinol-binding proteins in the white blood cells allows them to accumulate more retinol. This would imply, however, that cells which do not have these proteins (129) would not be protected (unless there exists some esterification-dependent protection) from accumulation of toxic retinol levels in their membranes. In addition, (iv) cellular retinol-binding protein levels were found not be the major determinants of retinol uptake and esterification in various squamous carcinoma cell lines (130). (v) If dissociation of retinol from RBP and transfer to the PC vesicles is rapid, then a significant amount of the serum retinol may be expected to partition into the lipoprotein (and albumin?) fraction of plasma and eventually spontaneously transfer to cell membranes. Such a spontaneous transfer of retinol between lipid vesicles and/or membranes has been observed in vitro (78, 81, 82). However, physiologic lipoproteins have been found to contain little free retinol while most of the plasma RBP is saturated with retinol (69, 82). Finally (vi), specific binding of RBP (with or without TTR), internalization of RBP, and internalization of TTR have each been suggested by separate studies involving different cell types or cell membrane preparations (69, 127, 129, and refs therein). Taken together, these results indicate that many cells obtain retinol through a selective, specific, possibly receptor-mediated mechanism.

Studies with testicular Sertoli cells (6), intestinal epithelial cells (131), ocular pigment epithelial cells (132-135), and placental brush border

membrane vesicles (136) have all documented competable, saturable binding of holo-RBP. Concomitant internalization of the RBP was not found in any of the (intact-cell) studies. In three of the studies, TTR added in the retinol uptake assays was found to decrease retinol accumulation rates by ~30% (6, 131) and ~40% (136). These studies suggest a mechanism for cellular retinol uptake which involves a cell surface RBP receptor that competes with TTR for the RBP-TTR complex. One possibility is that the receptor positions the holo-RBP close to the membrane and may facilitate the dissociation of TTR and retinol. An analogous system has been proposed for cholesterol uptake (or efflux) by cells from high density lipoproteins (HDL), where a cell surface protein is believed to anchor but not internalize the HDL (137, 211). Entry of the retinol into the cell may be facilitated directly by the receptor or it may occur simply by a combination of diffusion through the membrane and intracellular retention. In placental brush border cells, the RBP receptor is likely to be a protein because it is sensitive to trypsin and various reagents which modify proteins (136).

In contrast to the above results, which suggest that retinol but not RBP is internalized, in vivo studies (128, 138, 139) indicate that RBP is internalized by liver and kidney cells. When rats were injected with holo-RBP, labelled in either the protein or retinol moiety, uptake of holo-sRBP into liver cells was suggested (128). However, a separate study employing cultured liver parenchymal cells showed no significant uptake of labelled RBP; but labelled retinol delivered as a complex with RBP was taken up by the same cells (127). Considering that in the latter study (127) RBP was iodinated by a different method from the former (128) study, and that relative in vivo uptake by liver vs. kidney in the two studies (see also Ref.

140) was different, it is likely that the different RBP-labelling methods used can affect the results. Labelling (iodination) of RBP may affect physiologic RBP uptake by altering the interaction of RBP with (i) TTR (see Section IV.E and 141), (ii) retinol, or (iii) with the RBP receptor (cf. 142). In case (ii), the proportion of holo- vs. apo-RBP in the final labelled-RBP sample may affect RBP uptake.

Immunoelectronmicrographs of liver parenchymal cells at various times after the injection of a large amount of RBP showed the RBP to be present, along with a known marker of receptor-mediated endocytosis (asialo-orosomucoid) in vesicles near the cell surface at early times post injection, and deeper in the cytosol at later times (128). This was taken as evidence for receptor-mediated endocytosis of RBP. However, detection in clathrin-coated pits or vesicles was not shown, and it was not excluded that the observed vesicles were biosynthetic vesicles undergoing exocytosis (cf.143). Recently, no evidence for an RBP receptor in cultured hepatic parenchymal cells could be found (127); retinol uptake could be effected with the same uptake characteristics by RBP or two other proteins that can bind retinol, cellular retinol-binding protein and ß-lactoglobulin (127).

The uptake of transthyretin was not investigated in any of the internalization studies mentioned above. However, in cultured human hepatoma cells (HepG2) and rat primary hepatocytes, purified TTR showed saturable, high affinity binding (144). In the case of HepG2 cells, internalization was measured and was found likely to occur by a receptor-mediated mechanism (144). The effects of RBP-TTR complexation on binding and internalization of the TTR were not tested in that study.

Recently a putative RBP receptor from bovine retinal epithelial cells has been identified as a high MW complex (134). Because these cells are known to take up retinol from RBP but not the RBP moiety itself, the authors suggest that the receptor could transiently bind RBP and allow the retinol to pass into the cell. This complex has not been further characterized in terms of retinol uptake, but a putative subunit (p63) of it has been cross-linked to RBP (134). A monoclonal antibody against the subunit reveals that some p63 is on the cell surface, but a major fraction is present in intracellular membranes (134). Molecular studies and hydropathic analysis of the amino acid sequence (derived from a cDNA) suggest that p63 is not an integral membrane protein (135). Furthermore, expression of p63 in Hela cells does not reconstitute any RBP-binding activity (135). Thus, the role of this putative RBP receptor in retinol uptake is not clear. Even if p63 is a component of a bona fide retinol membrane-transport system, its importance appears to be restricted to retinal pigment epithelial cells; it could not be identified by immunoblotting and northern blotting in other tissues (134, 135).

D. SPECIFIC OBJECTIVES OF THESIS

In this project, an avian oogenesis system is employed as a model to provide (i) a molecular characterization of circulatory retinol transport, (ii) an understanding of oocytic retinol uptake mechanism(s), (iii) an analysis of oocytic endocytosis and yolk deposition of carrier proteins for nutrients and regulatory molecules (e.g., the retinol-RBP-TTR complex), and (iv)

information on how estrogen affects hepatic synthesis and serum levels of these carrier proteins, especially RBP.

It will be important to establish how retinol is stored in the yolk, and whether any protein components involved in retinol storage are locally synthesized in the ovary and/or derived from the circulation. If an oocyte membrane receptor/transporter for retinol, RBP, or TTR is identified, it will be of interest to clone it and analyze its structure and mechanism of transport. At present the only avian oocyte receptor system which is well characterized is that for the lipoproteins, VLDL and VTG. Any additional receptor system identified in this study may be used as a tool to probe the mechanisms of receptor-mediated endocytosis, a process which is poorly understood in oocytes.

Fig. I.1 Morphology of the ovarian follicle. The upper panel shows a schematic cross section of a typical chicken ovarian follicle. Nutrients from the blood pass through capillaries near the basal lamina and are available to the cells of the follicle, including the oocyte. The lower panel shows an electronmicrograph of a ~5 mm diameter follicle in cross section (lower corner represents the oocyte). The periplasmic space between the oocyte and granulosa cells contains many dark-staining bodies of unknown nature (Magnification, 3,500X).

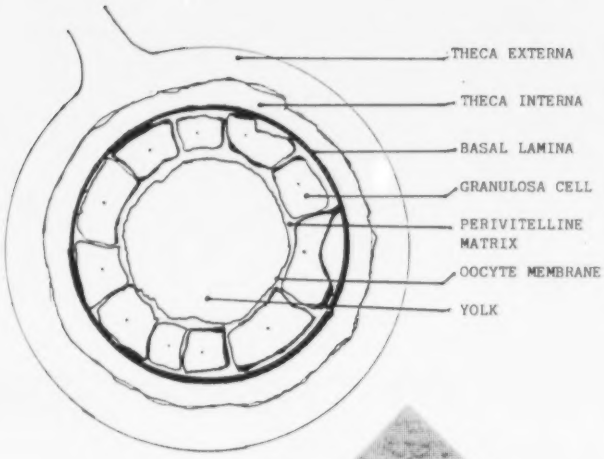

THECA EXTERNA
THECA INTERNA
BASAL LAMINA
GRANULOSA CELL
PERIVITELLINE MATRIX
OOCYTE MEMBRANE
YOLK

Fig. I.2 Overview of oogenesis in the chicken. Yolk precursors synthesized by the liver are transported via the circulatory system to the rapidly growing oocytes in the ovary. Upon completion of growth, the oocyte (commonly known as "egg-yolk"; dark circles in the figure) ruptures from its ovarian follicle and enters the oviduct. In the oviduct the albumen (commonly known as "egg-white") components and shell are added.

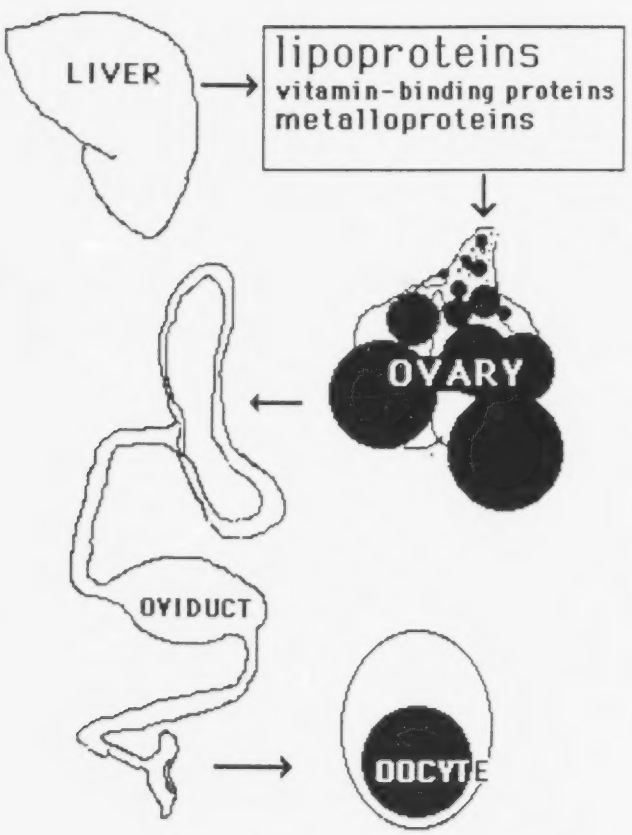

LIVER
lipoproteins
vitamin-binding proteins
metalloproteins
OVARY
OVIDUCT
OOCYTE

Fig. I.3 Schematic of the general structure of a lipoprotein particle. A neutral lipid core (dark shade) is sorrounded by a monolayer of polar lipids (unshaded), apolipoproteins (spotted structures), and possibly other proteins (not shown) such lipid transfer particles and the enzyme LCAT. This structure is applicable to Chylomicrons, VLDL, LDL, and some forms of HDL.

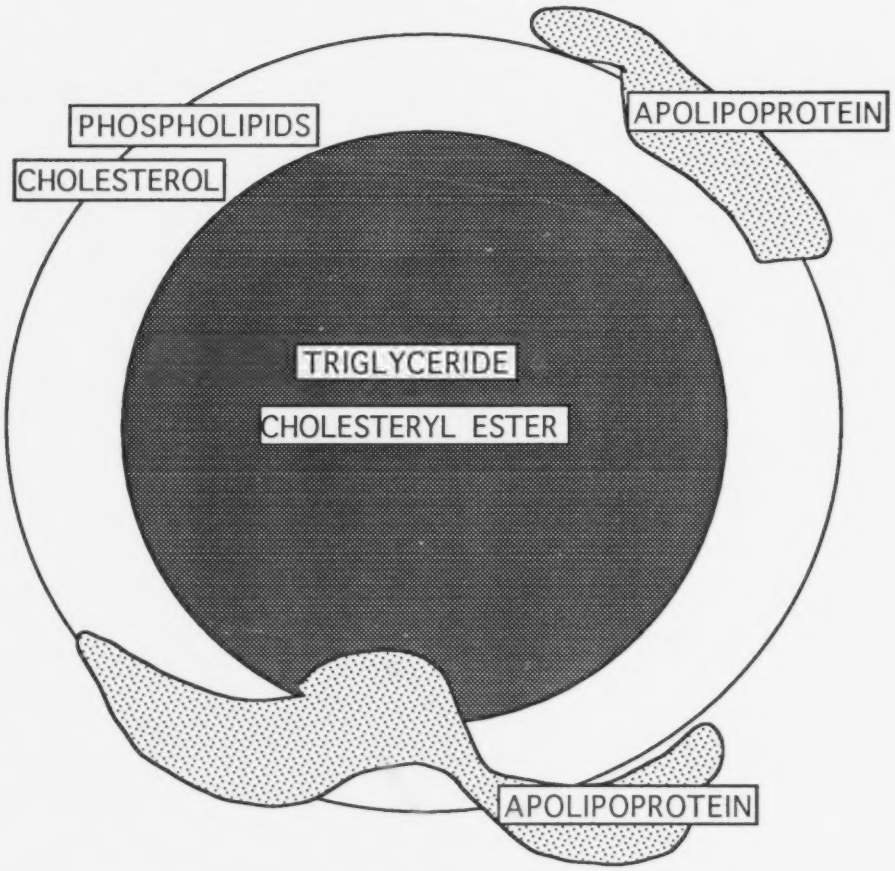

PHOSPHOLIPIDS
CHOLESTEROL
APOLIPOPROTEIN
TRIGLYCERIDE
CHOLESTERYL ESTER
APOLIPOPROTEIN

Fig. I.4 Overview of isoprenoid metabolism and retinol formation. Most cells possess the ability to synthesize sterols, geranylgeranyl pyrophosphate, and other important isoprenoids such as dolichol. However, only cells of photosynthetic organisms can synthesize carotenoids from geranylgeranyl pyrophosphate. Animals can convert certain dietary carotenoids such as ß-carotene to retinol. Retinol is metabolized to biologically active retinoids in the animals cells.

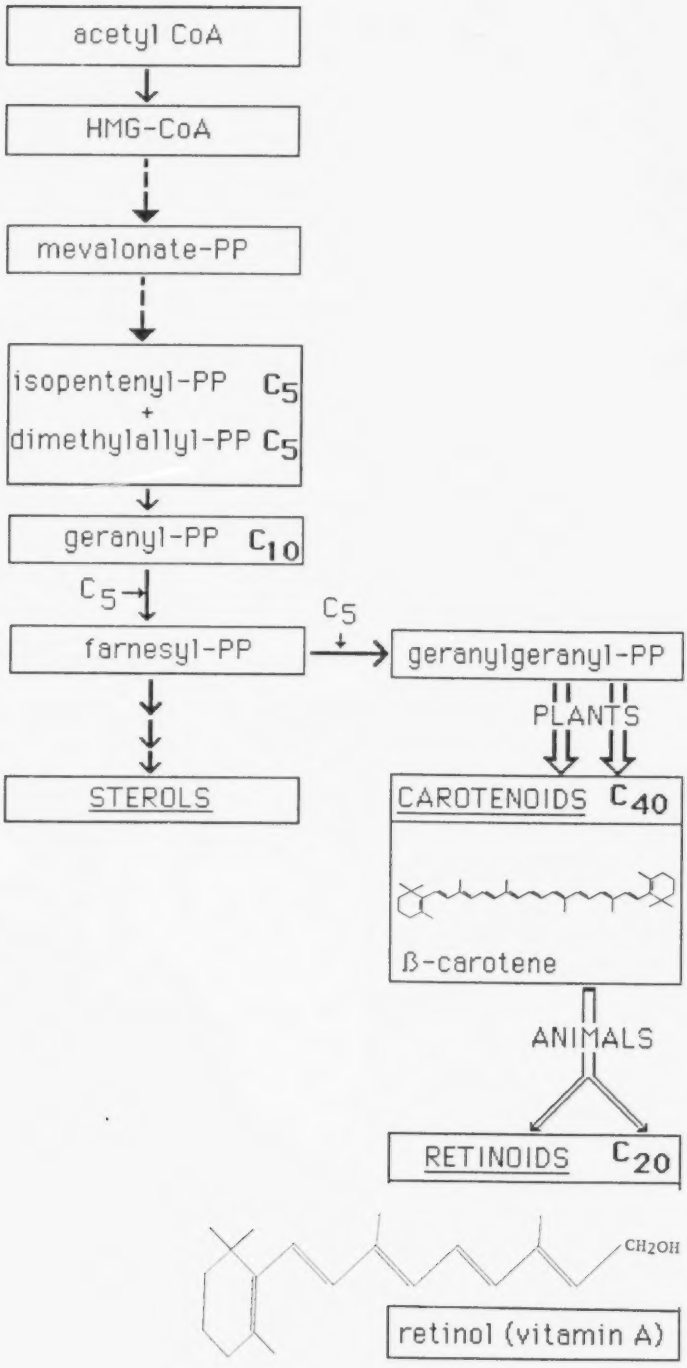

acetyl CoA
HMG-CoA
mevalonate-PP
isopentenyl-PP C5
+
dimethylallyl-PP C5
geranyl-PP C10
C5
C5
farnesyl-PP
geranylgeranyl-PP
PLANTS
STEROLS
CAROTENOIDS C40
ß-carotene
ANIMALS
RETINOIDS C20
CH2OH
retinol (vitamin A)

Fig. I.5 Transport and metabolism of retinol (R) is shown in this schematic summary. The only known physiological transport system for retinol in the circulation of vertebrates is retinol-binding protein (RBP). RBP forms a circulatory complex with a homotetrameric protein, transthyretin (TTR). The mechanism(s) of cellular retinol uptake from the RBP-TTR complex is not known. In mammals, its is known that circulatory retinyl esters (R-FA) and small amounts (nM) of retinoic acid (RA) can be transported by lipoproteins and albumin, respectively. Cellular activity, metabolism, storage and mobilization of retinoids is dependent on nuclear retinoid-binding proteins (e.g., RARs and RXRs), cytosolic retinoid binding proteins (e.g., CRBP), and enzymes such as retinyl ester hydrolase (REH), lecithin:retinol acyltransferase (LRAT), and acyl CoA:retinol acyltransferase (ARAT).

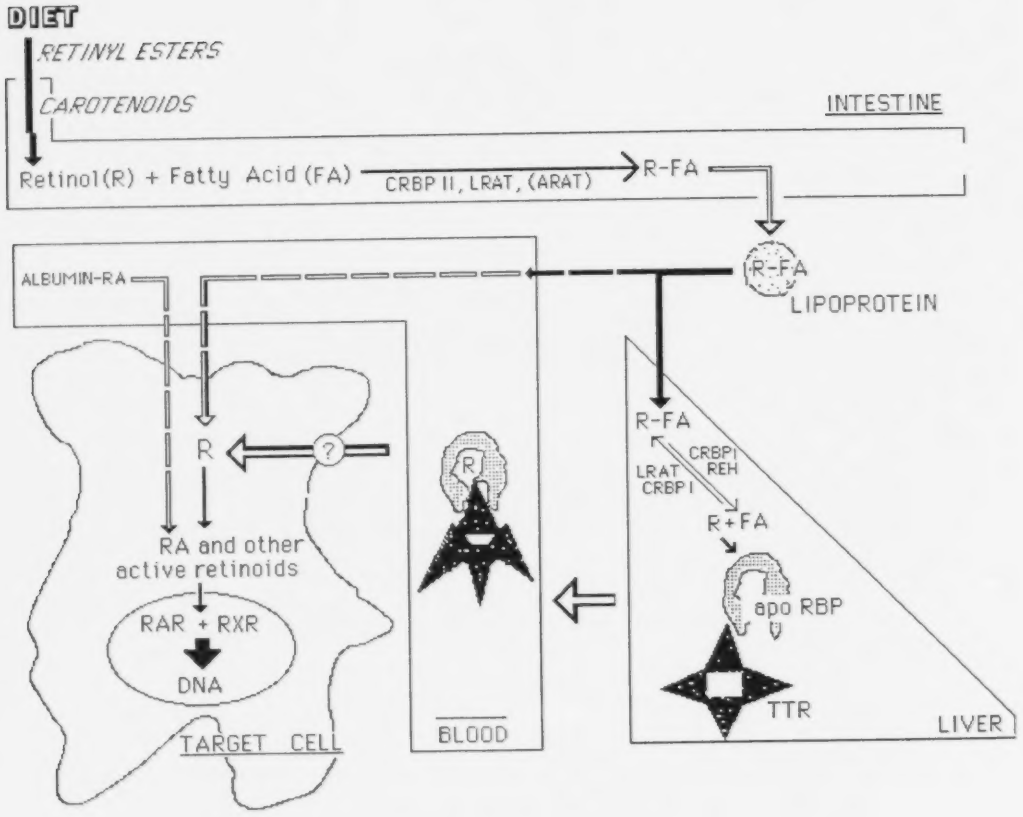
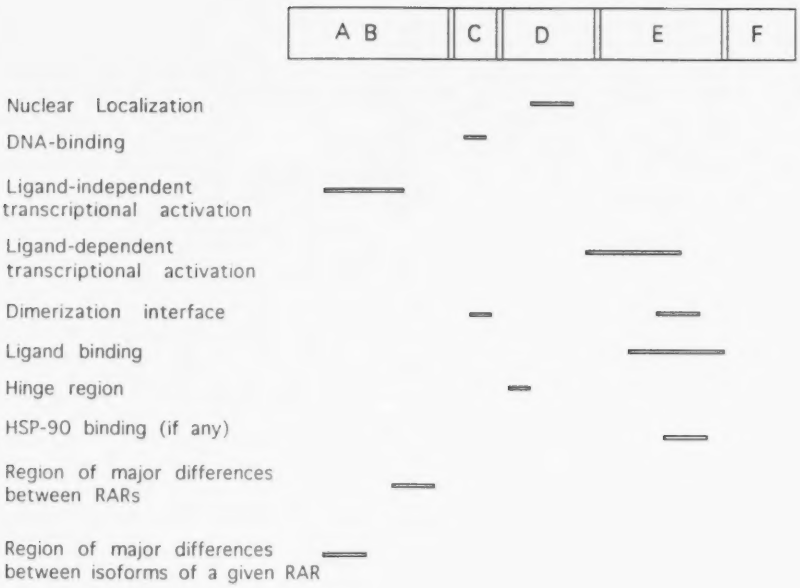

DIET
RETINYL ESTERS
CAROTENOIDS
INTESTINE
Retinol(R) + Fatty Acid (FA)
CRBP II, LRAT, (ARAT)
R-FA
R-FA
LIPOPROTEIN
ALBUMIN-RA
R
?
RA and other
active retinoids
RAR + RXR
DNA
TARGET CELL
BLOOD
R-FA
LRAT
CRBPI
CRBPI
REH
R+FA
apo RBP
TTR
LIVER

Fig. I.6 Structure and activity of nuclear retinoid-binding proteins. In the upper panel, a linear modular structure (A-F represent specific modules) along with several associated functions are shown for a typical member of the steroid/thyroid/retinoid nuclear receptor family. In the lower panel, a schematic model of nuclear retinoid receptor (RXR, RAR) function is shown. RXR and RAR, each containing one bound retinoic acid molecule, form a complex which interacts with two specific DNA retinoic acid response elements arranged as direct repeats (arrows). RXRs can also interact with other nuclear receptors such as the ones for vitamin D and thyroid hormone. The number (n) of bases (X) between the direct repeats dictates the types of heterodimeric complexes which can interact with the response elements. Other regulatory proteins (indicated by "?"; e.gs., the proteins "EA1" and "EA1-like activity") provide a functional link of the nuclear hormone receptors to the constitutive transcription machinery.


A B
C
D
E
F
Nuclear Localization
DNA-binding
Ligand-independent transcriptional activation
Ligand-dependent transcriptional activation
Dimerization interface
Ligand binding
Hinge region
HSP-90 binding (if any)
Region of major differences between RARs
Region of major differences between isoforms of a given RAR

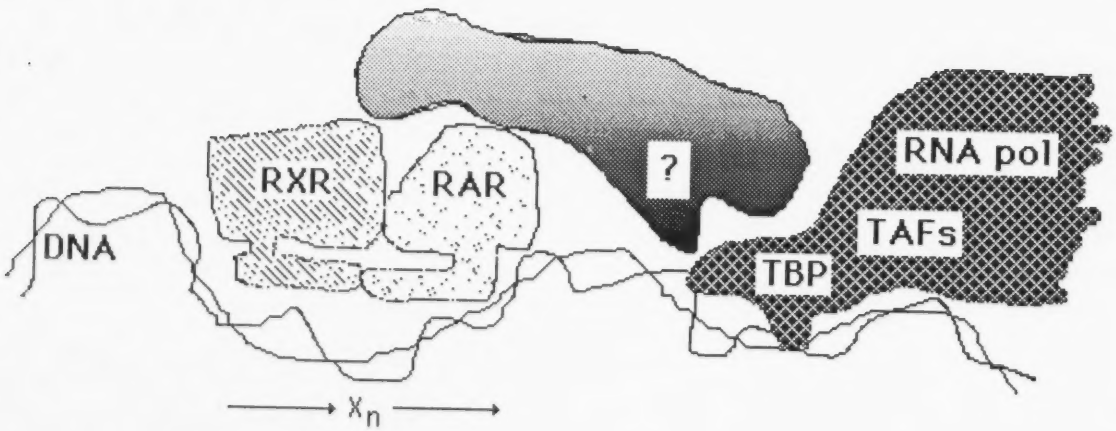
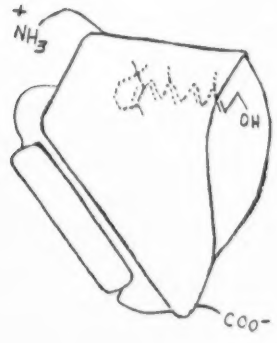

RXR
RAR
?
RNA pol
TAFs
TBP
DNA
Xn

Fig. I.7 The lipocalin protein family. Some lipocalins and their possible ligands are listed in the upper panel (for Refs. see Section I.3.a). In the lower panel, a schematic model of a lipocalin with bound ligand (in this case, RBP-retinol) is shown; this structural cup-like model is based on a similar schematic presented by Akerström and Lögdberg (TIBS 15, p. 241).

LIPOCALIN	LIGAND (ANIMAL SPECIES)
Plasma Retinol-Binding Protein	Retinol (vertebrates)
Apolipoprotein D	Steroids (?) (vertebrates)
Prostaglanding Synthetase	Prostaglandins (?) (vertebrates)
Bilin-Binding Protein	Bilin (insects)
Crusticyanin	Carotenoids (crustaceans)
Tear Prealbumin	Retinol (vertebrates)
p49 (?)	Unknown (bacteria)
alpha-1 Microglobulin	Unidentified Brown Chromophore (vertebrates)



CHAPTER II

EXPERIMENTAL PROCEDURES

A. MATERIALS

Enhanced Chemiluminescence- (ECL-) Western blotting detection kit, ECL-Hyperfilm, and horseradish peroxidase- (HRP-) streptavidin conjugates were obtained from Amersham. HRP-conjugated protein A, bovine serum albumin, chicken serum albumin, riboflavin-binding protein, rabbit IgG, chicken IgG, conalbumin, Freund's adjuvants, phenylmethyl sulphonylfluoride (PMSF), leupeptin, the crosslinker DSP (Dithio-bis(succinidyl propionate), Ponceau S, and the biotinylation reagent BNHS (N-hydroxy-succinimido-biotin) were obtained from Sigma; Centricon-10 microconcentrators from Amicon; nitrocellulose from Bio-Rad, Amersham, Schleicher & Schuell (special pore size 0.1 µm), and Hoefer Scientific Instruments; and M_r standards from Pharmacia, Sigma, Bio-Rad and BRL. Millex-PF filters (0.8 µm), protein A Sepharose, CNBr-activated Sepharose, and all Sephadex-based chromatography matrices were obtained from Pharmacia. Gold-labeled Protein A (15 nm particle size) was purchased from Janssen Life Sciences or E-Y Labs, and LR gold resin from Polysciences. 17ß-estradiol was obtained from Fluka. Purified human serum TTR was purchased from Calbiochem. DNA primers used in PCR and sequencing were obtained from the Department of Microbiology and Genetics, University of Vienna and the DNA synthesis laboratory, University of Calgary. The Iodo-Gen™ oxidative radiolabelling reagent was obtained from Pierce. Radioisotopes were obtained from Amersham, Edmonton Radiopharmaceutical, and DuPont. Anti-human RBP and anti-human TTR IgG was purchased from DAKO-immunoglobulins. Dialysis

membranes with a cutoff MW of 3500 and 6000-8000 were from Spectrum. Sources of other reagents are indicated below.

B. ANIMALS

White Leghorn laying hens, approximately 1 year old, and 1-5 months old roosters were obtained from the Institute of Molecular Pathology, Vienna, and the Department of Animal Sciences, University of Alberta. They were maintained with a 14 hour light period, and free access to food and water. Female New Zealand White rabbits (2-3 kg body weight) were used for antibody production.

C. IN VITRO TISSUE AND CELL CULTURE

1. Human HepG2 and Chicken LMH Hepatoma Cells

Unless otherwise stated, HepG2 cells (cell line provided by Dr. S. Yokoyama, University of Alberta) were cultured in Dulbecco's media (Sigma) + 10% fetal bovine serum (FBS) and 100 IU/ml penicillin, 0.1 mg/ml streptomycin. LMH cells (cell line provided by Dr. R. Binder, SUNY, Stony Brook) were cultured in Waymouth's media (Sigma) with the same additions as above + 1 mM glutamine. These hepatoma cells were cultured to near-confluency at 37°C in an atmosphere of 95% air, 5% CO_2.

2. Primary Chicken Granulosa and Embryonic Fibroblasts

Chicken ovarian granulosa cells were isolated according to a modification of an existing protocol (145). The granulosa cell layers isolated from the largest pre-ovulatory follicles (> 8mm diameter) were quickly placed into ice-cold, sterile medium A (140 mM NaCl, 5 mM KCl, 0.5 mM EGTA, 10 mM Hepes, 0.5 mM NaH_2PO_4, 0.5 mM Na_2HPO_4, 4 mM $NaHCO_3$, 5 mM glucose, 0.0006% phenol red, pH 7.4) and centrifuged at 200 x g, 4°C for 5 min. Cells were collected and washed twice (200 x g for 5 min) with medium A and then incubated in 10 ml of the same medium containing 500 ug/ml of collagenase (Type IV, Sigma) for 15 minutes at 37°C with constant shaking (100 cycles/min). Cells were collected as above and washed twice with medium B (minimum Eagle's media (MEM; Sigma) + 2 mM glutamine and antibiotics as above), and resuspended in 20 ml of medium B supplemented with 10% (v/v) FBS. Cells in suspension were counted and assessed for viability by trypan blue dye exclusion. Approximately 5 x 10^5 cells/ml (3 ml) were seeded in 60-mm dishes and cultured at 40°C under an atmosphere of 5% CO_2 and 95% air. Any experiments were initiated on day 6.

Fibroblasts were prepared from decapitated chicken embryos at day 15 of development essentially as described previously (210). The minced embryonic material was resuspended by repeated pipetting in TBS (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) + protease inhibitors + 0.25 % trypsin. Cells were washed and centrifuged to remove debris, counted and cultured, as above.

D. PREPARATION OF PROTEIN AND MEMBRANE SAMPLES

1. Plasma and Serum

Chicken blood was removed from a wing vein or directly from the heart by cardiac puncture. For plasma preparation, a coagulation inhibitor (EDTA or citrate) was added to the collection tube and the blood was centrifuged to remove cells. Serum was prepared by allowing the blood to clot (2-6 h, 4°C), followed by centrifugation to remove the clot.

2. Somatic Cell Membrane Extracts

For membrane extract preparation, monolayers were washed twice with 3 ml each of PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.5) and the cells were collected by scraping with a rubber policeman. Cells were lysed in 600 ml of a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM $CaCl_2$, 1.5% (v/v) Triton X-100, 1 mM PMSF, 2 uM leupeptin, and 2.5 mg/ml aprotinin. Lysates were centrifuged at 100,000 x g for 40 min at 4°C and the supernatants (solubilized-membrane proteins) were collected and stored frozen (-70°C). Alternatively, the cell membranes were prepared from the pelleted cells as described for the solubilization of oocyte membranes (see below) with the exception that the final Triton X-100 concentration was 1.4 % (v/v). Both of these methods were judged to be equivalent based on detection of the standard lipoprotein receptors.

3. Yolk

Yolk was collected after puncturing the separated "egg yolk" from an ovulated oocyte, or from the preovulatory follicle with care that the outflowing yolk did not contact any part of the external follicle. Typically, a mature, laid egg yielded 15 ml of yolk. For preparation of the yolk aqueous extract, each 10 ml aliquot of frozen yolk was thawed and mixed with 20 ml of ice-cold H_2O (or PBS) containing 1 mM PMSF and 2 uM leupeptin for 2 - 4 hr at 4°C. The mixture was then centrifuged at 20,000 x g for 25 min. The turbid supernatant was pooled, frozen at -70°C for 1 h and then thawed at room temperature. The thawed mixture was then recentrifuged (20,000 x g, 25 min) and the aqueous bottom phase was collected, filtered through 0.8 um Millex-PF and stored frozen. For the yolk HDL study, the aqueous extract was performed by dilution of yolk with 4 volumes of buffer (PBS or TBS) followed by two 10 min centrifugations (2 000 x g and 9 000 x g) and a final filtration performed as above.

4. Oocytic Membrane Extracts

All procedures were carried out on ice or at 4°C when possible. Ovarian follicles (2-10 mm in diameter) were placed in buffer (PBS or TBS, with the addition of 2mM $CaCl_2$, 1 mM PMSF, and 2 uM leupeptin) and cut free of thecal cell layers where possible. The yolk was extruded through an incision and the remaining material (basal lamina matrix, oocyte membranes and any adhering somatic cells) was repeatedly rinsed in the same buffer until the buffer became clear, i.e., free of yolk. Membrane fractions were obtained by mincing the material with scissors, homogenization (Polytron, setting 5 for 30 sec, and setting 8, twice for 20

sec) in ~5 ml of the buffer/g wet weight, and a centrifugation at 5000 x g for 5 min to remove debris. The resulting supernatant was then subjected to centrifugation at 100,000 x g for 1 hr (Fig. II.1). The membrane pellets were suspended in the buffer using a 18-gauge needle and syringe, and resedimented by centrifugation at 100,000 x g for 1 hr. The pellets were quickly frozen in liquid nitrogen and stored at - 70 ^{o}C for up to 4 months before use.

The follicular membrane pellets were solubilized as follows. Typically, each pellet obtained from the ovary of one chicken was resuspended with an 18-gauge needle in 1.5 ml 250 mM Tris-Maleate (pH 6.0), 2 mM $CaCl_2$, 1 mM PMSF, and 2 uM leupeptin. (In initial preparations, the suspension was sonicated twice for ~20 sec using a Sonifier microprobe at setting 6; but, this treatment was later found not to increase the amount of lipoprotein receptors that could be solubilized.) After the addition of 120 ul of 4 M NaCl the suspension was then passed through a 22-gauge needle a few times. Then, 150 ul of 20 % (w/v)Triton X-100 (final concentration 1% Triton; alternative detergents: 36 mM octyl glucoside or 30 mM CHAPS) was added to the mixture followed by resuspension through the 22-gauge needle and vortexing. The suspension was centrifuged at 100,000 x g for 1 hr, and the supernatant, membrane extract, was either used immediately or quickly frozen in liquid nitrogen and stored at -70°C for up to 6 months.

5. Oocytic Clathrin-coated Vesicles

Ovaries were dissected from laying hens and placed in ice-cold MES buffer (0.1 M 2(*N*-Morpholino)ethane sulphonic acid, 1 mM EGTA, 0.5 mM $MgCl_2$, 3 mM NaN_3, 1 mM PMSF, 5 uM leupeptin, pH 6.5).

Follicles of ~2-8 mm diameter were removed from the ovary and cut free of thecal cell layers where possible. Coated vesicles were prepared according to a low-sucrose method (146). Except where otherwise noted, all procedures were performed at 4°C. After two 20 sec homogenizations (Polytron, setting 5-6) of the pooled oocytes in two volumes of MES buffer, two centrifugations were performed to remove debris (5,000 g, 5 min) and then to pellet membranous structures (100,000 g, 1 hr). A similar centrifugation protocol was followed after resuspending the pellet in 6 ml of MES buffer. The second pellet was then resuspended again with 6 ml MES buffer, centrifuged (10,000 g, 10 min) to remove debris, and loaded on top of an equal volume of 6% (w/v) sucrose dissolved in D_2O in an Ultraclear™ Beckman tube. After centrifugation in a swing out rotor (80 000 g, 2 hr, 20°C), the solution was removed from the tube and the small pellet resuspended in MES buffer (100 ul for each ovary used). A final centrifugation (20 000 g, 10 min) to remove aggregates was performed and the pellet was stored frozen (an aliquot was kept at 4°C for structural analysis). Vesicles prepared in this fashion have been characterized both structurally and biochemically, and have been shown to be true clathrin-coated vesicles with fewer than 2% of non-coated vesicular structures (14, 53).

E. PURIFICATION OF PROTEINS AND LIPOPROTEINS

1. Retinol-binding Protein and Transthyretin

In a typical preparation, 25 ml of laying hen serum was mixed with an equal volume of 2x PBS (280 mM NaCl, 6 mM KCl, 16 mM Na_2HPO_4, 30 mM KH_2PO_4), pH 7.5, containing 1 mM PMSF and 2 uM leupeptin. The mixture was then centrifuged at 1500 x g for 5 min, filtered through Millex-PF, and applied to a DEAE-Sephadex A-50 column (15 x 3 cm) that had previously been equilibrated with PBS. The serum was passed over the column 3 times, followed by a wash with 50 ml PBS. Subsequent washes were performed, first with 30 ml of CBS (25 mM sodium citrate, 50 mM NaCl), pH 5.1, then with 30 ml of CBS, pH 4.1; and finally the elution was begun with 2x CBS, pH 4.1. Fractions of 3-8 ml were collected and tested for fluorescence at 463 nm, with an excitation wavelength of 330 nm. Fractions displaying high fluorescence were pooled and adjusted to pH 7.2 with 1 N NaOH. The neutralized solution was then passed 3 times through an anti-TTR IgG-Sepharose column (8 ml bed volume). The antibody column was washed with 100 ml of PBS. The bound RBP-TTR complexes were eluted with 100 mM glycine, 1 M acetic acid, pH 3; fractions (1-2 ml) were collected in tubes containing 100 ul of 1 M K_2HPO_4. Fractions with high A_{280} values were pooled, dialyzed against 5 mM glycine-NaOH, pH 10 in order to dissociate RBP from TTR, and then loaded onto a Sephadex G-75 superfine column (1 ml sample volume; 1.2 cm column diameter; 50 ml bed volume) that had previously been equilibrated with the same glycine-NaOH solution. Fractions collected after the void volume were analyzed by SDS-PAGE and Western blotting for the presence of RBP and TTR, respectively. The fractions containing these proteins were pooled separately, concentrated in Centricon-10 micro-concentrators, frozen and stored in aliquots at -20°C.

For the preparation of yolk RBP and TTR, the yolk aqueous extract was then subjected to chromatographies exactly as described for serum.

2. Liporoteins and Transferrin

Lipoproteins from both rooster and laying hen plasma samples were prepared using the same methods. For VLDL preparation, the plasma was first subjected to ultracentrifugation for 18 hr at 200,000 x g, 4°C. The floating lipoprotein fraction was mixed with 150 mM NaCl, 0.2 mM EDTA (pH 7.4), 1 mM PMSF, and 2 uM leupeptin (Buffer A) and a second centrifugation step at 200,000 x g for 24 hr was performed. Pure VLDL was recovered from the top of the centrifuge tube. The contents from the bottom half of the tube were then adjusted to a density of 1.060 g/ml with solid KBr, and centrifuged at 200,000 x g for 20 hr at 4°C. For LDL preparation, the top fraction resulting from this centrifugation step was dialyzed against buffer A and subjected to equilibrium density gradient centrifugation on a Buffer A-KBr density step gradient (bottom to top: 1.15 g/ml, 1.06 g/ml, and 1.02 g/ml; 3 ml each step) at 39,000 rpm at 4 °C for 12 hr in a Beckman SWTi 40 rotor. LDL was recovered at a position approximately 3 cm from the top of the tube. For HDL preparation, the bottom one third of the tube after the removal of VLDL and LDL (i.e., after the density=1.06 centrifugation) was adjusted to a density of 1.21 g/ml with KBr. HDL was then floated at 68 000 rpm (70.1 Ti rotor) for 36 hr, 4°C, and collected.

For the purification of transferrin, a 3-ml serum aliquot was thawed and mixed with 12 ml of buffer (1.5 M ammonium sulfate, 0.5 M sodium citrate, pH 6). The diluted serum was filtered through a 0.8 um Millex-PF filter and loaded onto a Phenyl Sepharose column (1.2 cm

diameter; 15 ml matrix volume) that had been equilibrated with the buffer. Chromatography was performed at room temperature. After loading the sample, the column was washed with 6 bed volumes of the buffer. Elution was begun with 0.75 M ammonium sulfate, 0.25 M sodium citrate, pH 6 by successive addition of 3-ml volumes and collecting the eluate. Protein absorption at 280 nm (A_{280}) was measured for each fraction. Starting with the first protein-containing fraction, 40 ul of each was analyzed by SDS-PAGE. The fractions containing the pure transferrin were pooled, dialyzed against PBS, and stored at -70° C.

F. FLUORESCENCE ANALYSES

Fluorescence emission of retinol, either free or complexed with protein, was monitored at 463 nm on a Hitachi F200 Fluorescence Spectrophotometer while varying the excitation wavelength. Alternatively, fluorescence emission spectra were obtained by holding the excitation wavelength at 280 nm or 330 nm and scanning the spectrum for the emission peaks. Further information is provided in Chapter IV.

G. GEL FILTRATION ANALYSIS OF YOLK AND SERUM

Serum (900 ul) or yolk aqueous extract (900 ul) was loaded onto a Sephadex G-100-fine column (48 ml bed volume, 5-6 ml/hr flow rate).

Fractions of approximately 1 ml were collected and analyzed for the presence of RBP and TTR by Western blotting. The median volumes of each fraction were used for determining K_{av} (see Fig. IV.2). In the case where the fractions were analyzed by fluorescence, a separate column was standardized as described in the legend to Fig. IV.1.

H. SDS-PAGE AND ELECTROBLOTTING OF PROTEINS

For SDS-PAGE and electrophoretic transfers, the Bio-Rad Protean or Mini-Protean vertical electrophoresis systems together with the respective Trans-Blot apparatus were used. Protein samples were mixed with an equal volume of loading buffer (20% glycerol, 4.5% SDS, 120 mM Tris-HCl, pH 6.8). Unless otherwise noted, polyacrylamide gradient gels (4.5 - 18%) were run in one dimension. After electrophoresis, the proteins were either stained (15% acetic acid, 38% isopropanol, 0.05% Coomassie Brilliant Blue) or transferred to nitrocellulose in 26 mM Tris, 192 mM glycine for 1-3 h at 200 mA.

I. ANTIBODIES AND IMMUNOBLOTTING

1. Antibody Preparation

Polyclonal antibodies were prepared by cutting the desired protein bands from gels after SDS-PAGE and a brief Coomassie staining.

The gel pieces were mashed by passage through a 1 ml syringe and incubated overnight at room temperature in 50 mM Tris, 250 mM NaCl, 2 mM $CaCl_2$, 0.1% SDS, pH 7.5. The proteins in the supernatant were collected after a brief centrifugation and precipitated with 4 volumes of ice-cold acetone for 1 h at -70°C. The precipitated proteins were then collected, dissolved in PBS and used for rabbit immunization (50-100 ug/injection). The IgG fraction was purified from rabbit sera on Protein A Sepharose CL-4B.

In the case of the anti-chicken RBP antibody, one of the rabbit injections was performed with RBP that had been isolated by chromatography, without the electrophoretic separation step. The resultant anti-RBP polyclonal antibody population crossreacted with chicken serum albumin as determined by Western blotting and competitions with purified chicken serum albumin (Fig. V.3 and 4). However, when the anti-RBP IgG was affinity-purified on immobilized pure RBP, the resultant antibody was immunopure and no longer recognized albumin (Fig. V.4). Antibody-Sepharose columns were prepared using CNBr-activated Sepharose (5-15 mg pure IgG/g dry beads), according to the manufacturer's procedure (Pharmacia).

2. Western Blotting

After SDS-PAGE, proteins were electroblotted onto nitrocellulose membranes and stained (0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid) for 10 sec to 3 min with gentle agitation. MW standards were marked, the nitrocellulose was cut, and the stain was removed with water and blocking buffer (blotto: 5% (w/v) non-fat milk, 0.5% (w/v) NaAzide, 1% (v/v) Triton X-100 in TBS). Membranes were

blocked in blotto by shaking for at least 2 hr at room temperature or overnight at 4°C. The blotto was replaced with a solution of the primary antibody (diluted 1:500 to 1:5000) in TBS-T (as blotto, except no milk or azide) or blotto. Antibody was incubated 1-2 hr or overnight as above. The membrane was then washed 3-5 times for 5-10 min in TBS-T, and the secondary antidody or protein A (1: 4000 dilution; either conjugated to HRP or labelled with ^{125}Iodine, 2uCi/ml) was added for a 30-60 min incubation with gentle agitation. Washes were performed in TBS-T as before. For ^{125}Iodine detection, the nitrocellulose was allowed to air dry and subjected to autoradiography at -70°C. For ECL detection (Fig. II.1), excess liquid was blotted off the membrane, and it was wetted on a plastic dish with a fresh mixture of the two ECL solutions (approximately 1 ml volume/20 cm^2 of membrane) for 1 min with a few flips of the membrane. Quickly the membrane was blotted to remove excess solution, wrapped with Saran™ wrap, and exposed to film for 5 sec to 20 min. ECL-blots were often prepared for reprobing by rinsing for a few hours with several changes of distilled water, or by treating with stripping buffer (60 mM Tris/HCl, 2 % SDS (w/v), 100 mM ß-mercaptoethanol, pH 6.7) for 30 minutes at 50 °C, and then placed in blotto.

J. PROTEIN LABELLING AND DETECTION OF LABEL

1. ^{125}Iodine

All procedures were done in the fume hood. Proteins and lipoproteins (dissolved in TBS or PBS) were labelled with Na^{125}I solution (~1 mCi) in a glass vial with a fresh, dry coating of the Iodo-gen™ oxidant

(100 ug in 100 ul of chloroform or acetone). After 10-20 min of reaction with gentle shaking, the labelled protein was subjected to repeated periods of dialysis until the counts per minute (cpm) in the dialysis solution were < 3,000. A protein measurement assay was performed on the labelled components and the specific activity was determined. Typically, the specific activities were as follows: vitellogenin, 50 cpm/ng; retinol-binding protein 300 cpm/ng; transthyretin 100-600 cpm/ng; low density lipoprotein (LDL) 100 cpm/ng.

2. Biotin

For whole serum biotinylation, BNHS (40 umol) dissolved in 1 ml of dimethylformamide was added to 5 ml of laying hen serum. After incubation for 1 hr at room temperature in the dark, the reactants were dialyzed at 4°C overnight in the dark against two changes of TBS. As a preinjection control, the biotinylated laying hen serum was blotted with streptavidin-HRP to confirm that a wide size-range of proteins had been biotinylated. Furthermore, it was confirmed that purified human and chicken TTR could be biotinylated under the same conditions used to biotinylate serum, and that biotin-TTR was still recognized by the anti-TTR IgG. Retinol-binding protein and transthyretin were biotinylated as above in 50-100 ul of PBS with 1ul of BNHS (38 nmol) dissolved in dimethylformamide. Molar ratios of protein to BNHS were approximately 1:20.

K. LIGAND BLOTTING

All blotting was performed at room temperature. Aliquots of the chicken oocyte or somatic cell membrane extract were subjected to SDS-PAGE (60-80 ug/ lane) under non-reducing conditions and electroblotted onto nitrocellulose. The nitrocellulose was blocked for 2 hr in 3% BSA (w/v) dissolved in TBSC buffer (50 mM Tris-HCl, 100 mM NaCl, 1mM $CaCl_2$, pH 7.5). Iodinated ligand (0.5-1 million cpm/ml of buffer) with or without an excess of unlabelled ligand was added to BSA-TBSC buffer and incubated for 2 hr. After 5 washes in TBSC over a period of 30 min, the nitrocellulose was allowed to dry and subjected to autoradiography. With biotinylated ligand, the basic procedure described above was used. The biotinylated ligand concentration was 0.1-0.5 ug/ml. The bound biotinylated ligand was probed with streptavidin-HRP followed by standard ECL detection (Fig. II.1).

L. PROTEIN CROSSLINKING AND IMMUNOPRECIPITATION

Chicken oocyte membrane extract (300 ul; 1.5 mg) and 30 ul (6×10^6 cpm; 10 ug) of I^{125}-TTR were added to 1.5 ml-Eppendorf tubes in the presence of 80 ul of PBS with or without 800 ug of purified human TTR. The tubes were incubated at room temperature with occasional mixing for 1 hr. DSP crosslinker (2ul; final concentration 1 mM) dissolved in DMSO was added to both tubes and allowed to react for 20 min on ice before quenching with 30 ul of 1 M Tris-HCl, pH 7.5. Purified preimmune rabbit IgG (33 ul; 350 ug) was then added to both tubes and allowed to bind for 3

hr before the addition of 100 ul of protein A sepharose (50% v/v suspension in PBS) for a further 1 hr. The tubes were then microcentrifuged at full speed for 20 sec and the supernatants were transferred to new tubes. The pellets were washed twice with 1% (w/v) Triton X-100 in PBS, first with 150 ul and then with 500 ul, by gentle vortexing followed by microcentrifugation as above. The final washed pellets are designated *non-immune precipitates*. Each of the first washes (150 ul) was combined with its respective supernatant and purified anti-chicken serum TTR IgG (33 ul; 360 ug) was added and incubated overnight at 4°C. The subsequent protein A Sepharose precipitation and wash steps were performed as above to yield the two final pellets designated *immune precipitates*.

The two *non-immune* and two *immune* precipitates were mixed with 150 ul of SDS-PAGE sample buffer and stored overnight at -20°C. To release the bound IgG and ligand complexes, the samples were heated at 95°C for 2 min and briefly microcentrifuged at maximum speed. The supernatants (60, 000 cpm from the immune and 11, 000 cpm from the non-immune reactions) were then loaded on a 4.5-18 % SDS-polyacrylamide gel and subjected to electrophoresis. The gels were subsequently stained, destained, dried, and subjected to autoradiography

M. IMMUNOELECTRON MICROSCOPY

Follicles were fixed (2% paraformaldehyde/0.2% glutar-aldehyde; 4°C, 1 hr) and processed for immunocytochemistry as previously described

(53). After blocking in 1% BSA, the mounted tissue sections were incubated with affinity-purified polyclonal rabbit antibodies against chicken serum RBP or TTR for 2 hr. Washes were performed with PBS. The tissue sections were then incubated with gold-labeled protein A, and stained with uranyl acetate and lead citrate (53). The tissue grids were examined with a Philips 300 electron microscope. Control sections incubated with preimmune rabbit serum showed negligible binding of protein A-gold.

N. IN VIVO PROCEDURES

1. Injection of Biotinylated Serum Proteins

The biotinylated serum was filtered through 0.8 um Millipore filters and then injected (4.5 ml) via a wing vein into the circulatory system of the hen. The hen was sacrificed 42 hr post-injection and the ovary transferred to ice-cold PBS. Working on ice over a period of 30-60 min, the follicles (1-3 cm diameter) were dissected from the ovary; each was punctured once with a needle, and the yolk allowed to flow into a clean vessel. Contact of the yolk with any external part of the follicle was avoided. The yolk was collected and pooled; and an aqueous yolk extract was prepared from it exactly as described (section II.D.3).

2. Estrogen Treatments

Roosters were injected into the leg muscle with 20 mg of 17ß-estradiol/kg of body weight or the equivalent volume of organic carrier, 1,2 propanediol. At various times post injection (6-125 hr), they were bled

from a wing vein and decapitated. A second estrogen treatment, 11 days after the first, was performed as above on one of the roosters. Serum proteins (section II.D.1), tissue RNA (section II.O), and liver cell nuclei (Section II.S) were prepared as described in the respective sections.

O. TISSUE RNA PREPARATION

Liver, kidney and adrenal tissues were immediately removed after decapitation and a small piece (<0.1 cm^3) of each was added to 1 ml of Trisolv™ solution (Biotecx). The remaining tissue was immediately frozen in liquid nitrogen and kept at -70°C for subsequent use. The tissues were homogenized on ice with a 15-ml hand-held Dounce homogenizer (Kontes) and the RNA was isolated according to the Biotecx protocol. The final RNA pellet was resuspended in TE buffer, quantitated by absorption at 260 nm and stored frozen (-20°C). Total RNA and poly A^+ enriched mRNA preparation from laying hen ovarian follicles was performed by a previously reported method (147).

P. NORTHERN BLOTTING

Each RNA sample was mixed with 10 ul RNA denaturing buffer containing 5 ug ethidium bromide, heated at 65°C for 15 min, and cooled on ice. RNA samples were electrophoresed at 100 V through an agarose gel

(1.2 g agarose dissolved in 86 ml H_2O, and mixed with 10ml 10X MOPS, 11 ml formamide before pouring) until the bromophenol blue band had migrated 3/4 of the way down the gel. After electrophoresis, the gel was soaked in 10X SSC for 10 min and blotted overnight with 10X SSC onto nitrocellulose (Amersham). The nitrocellulose was photographed under UV light and baked for 2 hr at 80°C. Prehybridization was performed in 5X SSC, 5X Denhardt's, 0.5% SDS and 400 ug denatured salmon sperm DNA for 4-16 hr at 65°C. The probes were labelled with alpha-^{32}P-dUTP using a random primer method (Megaprime™, Amersham) and purified on NICK™ columns (Pharmacia). For hybridization, the probe was denatured and added to prehybridization solution and incubated at 65 °C (or 42°C with 50% formamide) for 18-28 hours. The membranes were subsequently washed three times with 0.5-1X SSC, 0.1% SDS for 15 min at 50-65°C and rinsed two times with 0.5X SSC, 0.1% SDS. The above procedure was used with the chicken RBP cDNA probe as well as with a human TTR cDNA probe (gift from Dr. W. Blaner, Columbia University); however, the Northern blot was successful only in the former case. Northern blots at lower stringency (e.g., 50% formamide, 30°C) also failed to reveal any signal for the chicken TTR RNA.

Q. PCR ANALYSIS AND cDNA LIBRARIES

PCR reactions for the amplification of RBP cDNA contained Taq (Promega) or DyNA Zyme (Finnzyme) DNA polymerases, 0.5 uM primer A (GGX ACX TGG TA(TC) GCX ATG GC), 0.5 uM primer B (TA(GA) TCX

GT(GA) TCX AC(TGA) ATC CA), (where X is A, G, T, and C), and 2 ug of purified template DNA prepared from a chicken liver cDNA library (Clontech) in the bacteriophage lambda gt 11. A Perkin-Elmer DNA Thermal Cycler 480 was used with the following settings for 35-40 cycles: 94°C, 1 min; 40°C, 1 min; 72°C, 2 min. Various control reactions were performed by omitting the primer(s) or template. The expected RBP PCR product was isolated by elution from low melting point agarose (148) and subcloned using the pGEM-T vector (Promega). PCR was also performed as above with templated DNA from a chicken follicle lambda gt11 cDNA library. This library had been custom made (Clontech) by random priming from mRNA derived from chicken follicles (4-6 mm diameter) following surgical removal of thecal layers.

R. cDNA CLONING AND SEQUENCING

Screening of the lambda gt-11 chicken liver cDNA library (Clontech) was performed by the method of Benton and Davis (149) and the isolated chicken liver RBP cDNA (initially, 1 positive plaque/~50,000 plaques screened) was recloned as an EcoRI fragment in pBLUESCRIPT (Stratagene). Both strands of the RBP cDNA, and the RBP PCR product, were sequenced with synthetic oligonucleotide primers using the Sequenase™ version 2 kit according to the recommended protocol (USB). Outline of optional direct cloning strategy: the biotinylated DNA probe was annealed with cDNA library DNA (e.g., lambda gt 11 library). Avidin (or streptavidin) Sepharose was then used to precipitate the biotin-probe-

cDNA complexes. After extensive washes at high stringency, the non-biotin-DNA was released from the avidin matrix with NaOH (or heat), reannealed, digested with the appropriate restriction enzyme and subcloned into a plasmid for cell transfection. Positive clones were sequenced directly. This procedure was used for cloning cDNAs which are not included in this thesis.

S. RUN ON TRANSCRIPTION ASSAY OF THE RBP GENE

Approximately 27 hours post-estrogen treatment, the roosters were sacrificed, and the livers were removed and processed for the production of liver cell nuclei essentially as previously described (150). All procedures were carried out on ice. The nuclei were quantitated by counting under the microscope and nuclei pellets (4×10^{-7}/100 ul of solid pellet) were frozen in liquid nitrogen and then stored at -70°C.

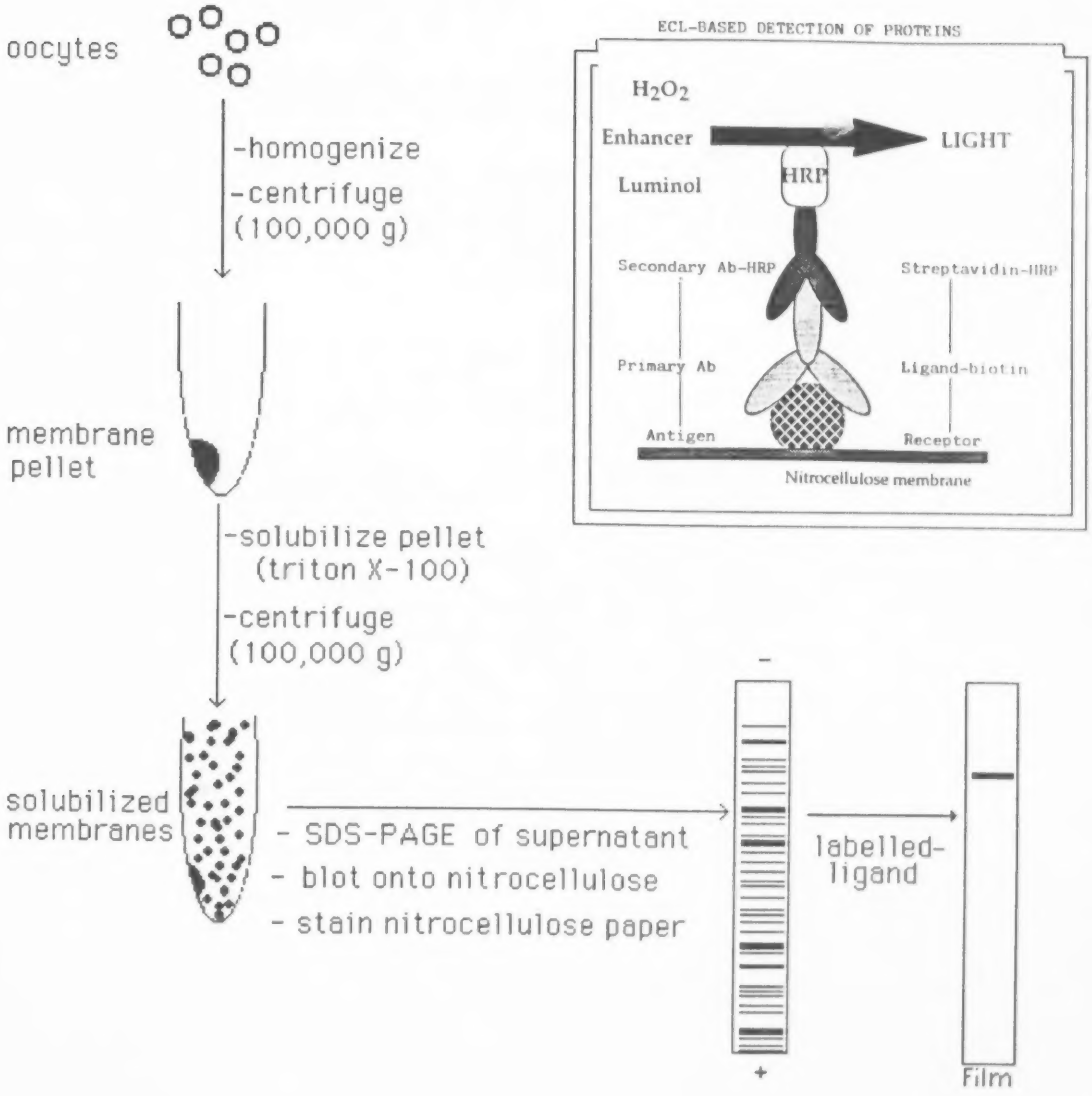
The run-on transcription assays were performed according to a previously reported method (151 and refs therein) using 4×10^{7} nuclei/assay. The nuclei were incubated for 30 min at room temperature in 200 ul of a buffer containing 87 mM Tris-HCl, pH 7.9, 24 mM NaCl, 200 uM EDTA, 1 mM DTT, 83 mM ammonium sulfate, 7mM $MnCl_2$, 50 uM PMSF, 200 ug heparin, 1 mM NTPs (AGU), 10 mM creatine phosphate, 20 ug creatine phosphokinase, 100 units RNasin (Promega), and 50 uCi ^{32}P-CTP. Subsequent digestions with RNase-free DNase I (Boehringer) and proteinase K were performed for 35 min at room temperature and 50 min at 42°C, respectively. After one phenol-chloroform-isoamyl alcohol (PCI) extraction to remove protein, the RNA transcripts were precipitated in the

presence of tRNA with an equal volume of 10% (w/v) TCA/60 mM pyrophosphate and separated from the free nucleotides by filtration through 0.4 um Millipore glass filters. The filters were treated with DNase and the transcripts were eluted and treated with proteinase K, 30 min at 37°C. Another PCI extraction was performed and the transcripts were subsequently treated with 1/5 volume of 1 M NaOH for 10 min on ice. The brief RNA digestion was stopped with 1/3 volume of 1 M HEPES free acid, and the RNA was precipitated with 1/10 volume of 3 M NaAcetate and 2 volumes of ethanol, overnight at -20°C. After a centrifugation at 2,200 x g for 35 min, the RNA pellet was washed with 70% ethanol and dissolved in 1 ml of hybridization buffer. The radioactivity was quantitated and the hybridization with the DNA (see Fig. III.4) was performed as in the Northern blot using 1 X 10^6 cpm/ml. Three washes were performed with 2X SSC for 10 min at 65°C, followed by an RNase A (15 ug/ml) digestion at 37°C for 40 min. Three final washes were performed in 2X SSC for 5 min at room temperature.

T. OTHER METHODS AND COMPUTER PROGRAMS

Proteins were quantitated by the method of Lowry et al. (152). The Geneworks™ program (IntelliGenetics; Feb. 1993 release) was used for analysis of the RBP DNA sequence and for aligning the various RBP and TTR protein sequences. A Personal Densitometer (Molecular Dynamics) was used for densitometric scanning along with the imageQuant™ computer program.

Fig. II.1 Illustration of the oocyte membrane ligand blot procedure, and (insert) of the ECL-based strategy for Western blotting and ligand blotting methods used in the experiments described in this thesis.


oocytes
-homogenize
-centrifuge
(100,000 g)
membrane
pellet
-solubilize pellet
(triton X-100)
-centrifuge
(100,000 g)
solubilized
membranes
- SDS-PAGE of supernatant
- blot onto nitrocellulose
- stain nitrocellulose paper
labelled-
ligand
Film
ECL-BASED DETECTION OF PROTEINS
H_2O_2
Enhancer
LIGHT
Luminol
HRP
Secondary Ab-HRP
Streptavidin-HRP
Primary Ab
Ligand-biotin
Antigen
Receptor
Nitrocellulose membrane

CHAPTER III

SYNTHESIS AND STRUCTURE OF CHICKEN RBP AND TTR

In order to gain further insight into the molecular properties of avian RBP, an RBP cDNA was cloned from a chicken liver lambda gt11 library. The DNA and deduced protein sequences of the clone, the first known avian RBP, revealed several unique structural properties relative to the other known RBPs. Use of the cDNA as a probe provided insight into the tissue distribution and hormonal regulation of the RBP mRNA. A similar analysis of synthesis and stucture is presented for chicken TTR.

A. CLONING AND STRUCTURAL ANALYSIS OF CHICKEN SERUM RBP

Both the primary and 3-dimensional crystal structure of human serum RBP as well as the primary structure of other RBPs and lipocalin RBP homologues are known. Based on the primary structure of known RBPs and other lipocalins, DNA primers (see Section II.Q and Fig. III.1) were designed and synthesized for the identification of avian RBP cDNA. The primers were designed to include the two most conserved lipocalin motifs, GTW and TDY, and to minimize degeneracy (Fig. III.1). The 7-amino acid sequences selected are separated by ~75 amino acids (~225 base pairs) in most lipocalins including RBP. The presence of RBP in the chicken liver lambda gt 11 library was initially demonstrated by PCR analysis using the lipocalin specific primers followed by DNA sequencing. PCR analysis of a chicken ovarian follicular library using the same primers failed to identify RBP but led to the identification of a putative, novel

lipocalin based on partial DNA sequence. Subsequently, a complete RBP cDNA was isolated from the liver library and sequenced. Figure III.1 shows the sequence of the chicken RBP cDNA isolated from liver. Salient features of the RBP mRNA deduced from the cDNA sequence include (i) two consensus polyadenylation motifs (AAUAAA; nucleotides 771-776 and 792-797) which are both immediately preceded by putative cytoplasmic polyadenylation elements (CPEs; UUUUAUU, nucleotides 764-770; UUUUAAU, nucleotides 788-794), (ii) multiple in-frame stop codons (nucleotide positions 622, 634, 655), the first of which gives rise to a protein 3-7 amino acids shorter than RBPs from other species, and (iii) five AU-rich, potential destabilization sequences (AUUUUA, nucleotides 709-714, 763-768, 787-792; AUUUA, nucleotides 724-728, 741-745) throughout the 3' UTR.

The deduced amino acid sequence of the chicken RBP was aligned (Fig. III.2) with that of the human protein to reveal homology in the primary structure and of the ß-strand elements which form most of the secondary structure. Over the entire length of the chicken RBP sequence (175 residues), 87% (152 of 175) of the residues are identical to those in human RBP. This high degree of similarity makes RBP one of the most highly conserved serum proteins known. With one possible exception, the presence of a known ß-sheet breaker, Glu122, close to the end of the seventh ß-strand, there are no amino acid substitutions in the chicken protein which would greatly disrupt any of the known secondary structures of human RBP. All residues which are known to lie within 0.4 nm of the retinol ligand in the human RBP (153) (Leu35, Phe36, Ala43, Phe45, Ala57, Val61, Met73, Gly75, Met88, Leu97, Gln98, His104, Tyr133, and Phe135),with the exception of Gln117, are also conserved in the

chicken protein (Fig. III.2). Two previously reported partial protein sequences of N-terminal fragments of chicken serum RBP, ERDXRVSXFKVKENFDKNRYSGTWYAMAKKDPEGLFLQDNVVAQFTV (154) and EXDXRVSSFKVXENFD (155) (where X is an undefined amino acid), are identical to the corresponding region in our clone and demonstrate that we have indeed identified the avian serum protein.

The chicken serum RBP contains two main features which are not conserved in any of the other known RBPs (human (156, 157), rat (158), *Xenopus* (150), rabbit (158) and a partially sequenced fish RBP (159)): (i) an unusually short C-terminal sequence with only one amino acid after cysteine 174, and (ii) the presence of two extra negative charges (Glu122 and 125) in the short disulfide-bridged loop between the end of the seventh ß-strand and the beginning of the eighth (see Fig. III.2, Cys120 to 129). It is not known to what extent, if any, these differences affect the structure of the protein and, in particular, the stability of proximal disulfide bonds. The six cysteines in the chicken RBP are conserved, and it has been shown (153, 159, 160) that in other RBPs they are disulfide-bonded to each other. As judged from the large decrease in electrophoretic mobility upon SDS-PAGE under reducing conditions (cf. Fig. IV. 8), the structure of chicken RBP is also highly compacted by disulfide bonds. I found that a small fraction of chicken RBP, however, was disulfide-bonded to an unidentified high molecular weight serum protein. Such a complex has not been reported for any of the other known RBPs. The physiological relevance of this association is questionable because it is almost completely abolished if blood is collected in the presence of a reagent (e.g., DTNB) that reacts with free -SH groups; however, this association may indicate a slight difference in the reactivity of at least one of the disulfide bonds (or -SH groups) of the

chicken RBP relative to the other known RBPs, e.g. increased structural strain of the bond or surface exposure of the oxidized or reduced sulfhydryl. In this context, another member of the lipocalin protein family, human apolipoprotein D, has recently been shown to form disulfide-bonded heterodimers with a high molecular weight serum protein, apolipoprotein B (161).

B. CHICKEN TISSUE RBP mRNA LEVELS AND THE EFFECTS OF ESTROGEN

To analyze the relative abundance of RBP mRNA in various tissues of the chicken, Northern blotting was performed using the ^{32}P-labelled chicken RBP cDNA. Because the known serum-to-yolk transport components are strongly induced by estrogen at the hepatic RNA level (25-28), I analyzed whether RBP was also subject to estrogen induction. For this, roosters were injected with 17ß-estradiol and, at various post-injection (p.i.) times, total mRNA from tissues was prepared.

Liver from both control and estrogenized roosters contained an RBP RNA of approximately 1 kbp (Fig. III.3A). No RBP RNA could be detected in kidney (or adrenal gland). An RBP mRNA of similar size to that of chicken has been reported in human (156), rat (162) and *Xenopus* liver (150). Estrogen treatment led to an initial decrease of the RBP RNA signal relative to the controls (Fig. III.3A and B). At a later time (81 hr) post-estrogen treatment (p.i.), the RBP RNA levels increased but were still below control levels (Fig. III.3B, lane 2). A time course obtained by

densitometric scanning of Northern blots such as the ones presented above shows the quantitative changes in RBP, apoVLDL II and actin RNAs as function of time post estrogen treatment (Fig. III. 3C). A second estrogen treatment, 11 days after the first, also did not reveal any induction of the RBP RNA; in fact, the observed RBP RNA signal after this second treatment (Fig. III.3B, lane 3), which represents 49 hr p.i., was intermediate in intensity between the 26 hr and 81 hr signals in Fig. III.3B, and represents a value of 5.4 in Fig. III.3C. The efficacy of the estrogen administration was tested by analyzing the apolipoprotein VLDL II RNA levels (and vitellogenin serum protein; see Appendix A); they were shown to undergo the expected (17, 18) dramatic inductions (Fig. III.3B and C). Actin RNA levels, provided as another control, show little change upon estrogen treatment (Fig. III.3B and C).

As shown in Figs. III.3B, lane 1 and III.3C, the maximal estrogen-dependent decrease of RBP RNA occurs at approximately 20-30 hr p.i.. To obtain some insight into the possible causes of this decrease, liver nuclei were prepared from control and estrogen treated roosters (27 hr p.i.) for run-on transcription analysis of the RBP gene. The results reveal an acute estrogen-dependent decrease of RBP gene transcription (Fig. III.4).

The above experiments provide an important comparison of the regulation of RBP with that of other known yolk precursors such as the lipoproteins, VTG and VLDL. Estrogen induction of the yolk precursor lipoproteins at the hepatic RNA level is well established (17, 18, 20, 21, 23, 163). In contrast, the hepatically synthesized chicken RBP mRNA is not induced, and even shows a decrease after estrogen treatment relative to controls. Interestingly, the kinetics of this decrease (6-16 hours lag time) are similar to those reported for the *increase* of vitellogenin mRNA in estrogen-

treated roosters (22). We show that one cause of this reduction in RBP RNA is an estrogen-dependent decrease of RBP gene trancription (Fig. III.4). Such changes in RBP RNA levels and gene transcription, however, are possibly an effect of the acute estrogen-treatment of roosters (perhaps due to the hormone's immediate induction of hepatocyte recruitment and differentiation; Refs. 168, 209), and may not affect retinol transport in the laying hen which is subject to chronic estrogen exposure and whose circulatory RBP levels do not change significantly relative to roosters (Appendix A).

Analysis of the 3' UTR of chicken RBP mRNA reveals that it contains unique motifs relative to the other known RBP mRNAs: (i) putative AU-rich destabilization sequences, including two AUUUA pentamers for which a specific destabilizing binding protein has been identified (165), (ii) multiple in-frame stop codons, (iii) two polyadenylation signals, both of which are preceded by (iv) U-rich sequences which conform to the consensus of known CPEs. CPEs have been proposed to function along with the polyadenylation signal in the regulation of the mRNAs' poly(A) tail length (reviewed in 167). These features suggest multiple levels of control for the RBP mRNA which are unique to the chicken (or to avian species). The results showing that acute estrogen treatment of roosters decreases RBP gene transcription in the liver do not exclude additional estrogen-dependent changes in RBP mRNA stability, or mRNA processing and export from the nucleus. It remains possible that regulatory elements in the 3' or 5' UTR of the RBP transcript have a role in the estrogen response observed. To my knowledge, the only other serum protein in an oviparous species whose mRNA levels have been shown to be *decreased* by estrogen is serum albumin (SA), in both

Xenopus and chickens (23, 164). In the case of *Xenopus* SA, estrogen induces a destabilization of the mRNA which is independent of protein synthesis (164). By analogy to results in other systems (165, 166), one possible explanation for the observed decrease in SA mRNA is that acute estrogenization leads to its destabilization due to post-translational regulation of a factor that interacts with the 3' UTR of the mRNA. In this context, we have noted that both RBP and SA mRNAs share 75% identity in the last 35 base pairs of their 3' UTRs. The RBP and SA estrogen responses are, however, not expected to be completely identical because in the latter case (cf. Ref. 23) but not the former (Chapter V) the serum protein levels are greatly decreased. The estrogen response which we observe appears to be an unique feature of chicken RBP. In *Xenopus*, estrogen is known to dramatically induce hepatic RBP RNA and gene transcription levels (150, 168); in the rat, estrogen treatment induces RBP RNA levels in the kidney, but not the liver (169). Thus, a comparative analysis of the molecular mechanisms which underlie these differences may be helpful to further understand the function of the estrogen hormonal system.

Without knowledge of the regulatory cis-elements (e.g., estrogen response elements, EREs) in the RBP gene, it is difficult to speculate on possible mechanisms of acute, estrogen-dependent RBP gene repression. Examples of transcriptional repression due to estrogen and its nuclear receptor (ER) are rare relative to examples of activation (212). Possible mechanisms of estrogen-dependent transcriptional repression include: (i) competition of nuclear receptors for overlapping or interacting DNA REs (reviewed in 212, 213), (ii) the existence of activation and repression REs, e.g., REs with a slightly different sequence (reviewed in 212), (iii) competition of estrogen-ER with another factor required for transcription

of the repressed gene (212-214), (iii) post-translational modification of a repressor or activator (98, 212, 215), (iv) induction of other regulatory factors which are involved in transcriptional control of the repressed gene.

Previous observations suggest that most yolk components are derived by uptake from the blood compartment and are not synthesized by the oocyte or other ovarian follicle cells (13, 14, 47-49, 53). To provide more conclusive evidence concerning RBP in this respect, total RNA and mRNA was isolated from the laying hen ovarian follicles (i.e., oocyte and adherent somatic cells) and analyzed RBP expression by Northern blotting. The expected 1 kbp message was detected in 10 ug liver RNA (Fig. III.5, lane 1); however, as shown in Fig. III.5, lanes 2 and 3, no detectable RBP message was found in 20 ug of total RNA (or in 5 ug of poly A^+ enriched mRNA, not shown) from follicles of various sizes. Consistent with this observation, the same lipocalin-consensus oligonucleotide primers which identified RBP in the chicken liver library failed to identify RBP in a chicken ovary library by PCR analysis. Thus, these results suggest that no significant levels of RBP are expressed in the ovarian follicle, and support the hypothesis of the extrafollicular (hepatic) origin of yolk RBP.

C. STRUCTURE AND SYNTHESIS OF CHICKEN TTR

The 3-dimensional crystal structure is known for normal human TTR (170) as well as for a mutant TTR involved in familial amyloidic polyneuropathic disease (171). I initiated the cloning of chicken serum TTR (and RBP) with the ultimate goal of analyzing its estrogen regulation and

possible sites of interaction with RBP by mutation/expression experiments. Before completion of cloning, however, the full sequence of chicken TTR as well as a 3-dimensional model of its structure based on the known human TTR 0.18 nm-structure was reported by another group (172). The tertiary and quaternary structures of the human and chicken proteins were predicted to be highly similar (172). The amino acid sequences of chicken (130 amino acids) and human (127 amino acids) TTRs are 78% identical overall (Fig. III.6), and completely identical in a central channel formed by the tetrameric assembly of the monomers(172). TTR can bind the thyroid hormones, T_3 and T_4, directly in this central channel (170, 173, 174). In light of the high homology between the human and chicken TTRs, it is surprising that a polyclonal antibody against the chicken protein (raised in rabbit) does not recognize its human homologue, and vice versa (Fig. III.7). Thus, some of the amino acid differences between the human and chicken protein are likely to be part of the most antigenic epitopes.

One unusual feature of the TTR structure is its great stability over wide pH ranges and various concentrations of denaturants (125 and refs therein). The TTR dimer is even more stable than the tetramer; noncovalent dimeric TTR structures (188, 189) are observed upon SDS-PAGE without sample heating (e.g., Fig. IV.7). Minor amounts of dimer can even be observed by Western blotting after some heating (e.g., Fig. IV.6 and Fig. III.7). In general, TTR dimers are observed upon SDS-PAGE with sample heating times of <5 min at 95 degrees C.

An analysis of the relative abundance of TTR RNA in various tissues of the chicken suggests that, like RBP, the TTR message is abundant in liver and, to a much lesser extent, some other tissues such as eye and lung (172). The relative contributions of gene transcription and RNA decay

rates were not determined, however; and it is, thus, not possible to exclude these factors from the relative steady state abundance levels reported for each tissue. Unlike the case of RBP, however, the choroid plexus of the brain also synthesizes TTR. In vertebrates including the chicken, the choroid plexus is the major site of TTR synthesis (~30-fold greater than liver/g of wet tissue) (172). It is believed that the choroid plexus TTR has an important role in the delivery of the thyroid hormones required for normal adult function of the brain and during its embryonic development (172 and Refs therein). In the case of the chicken ovary, no information is yet available on possible TTR expression (see also Section II.P).

Fig. III.1 Nucleotide and amino acid sequence of chicken serum retinol-binding protein. An RBP cDNA clone was isolated from a chicken liver lambda gt 11 library and sequenced. The presumptive signal sequence is shown in lower case; and the mature protein (known from protein sequencing, see text) is shown in upper case, amino acid single letter code. Two 7-amino acid sequences used for synthesis of the PCR oligonucleotide primers are underlined. The multiple in-frame stop codons are doubly underlined. Two nucleotide hexamers representing polyadenylation signals are overlined. Other features of the nucleotide sequence are described in Section III.A. This sequence is available from the EMBL protein data bank with the accession code X77960.

```
GAATTCCGCAGGGAGCGGAGCTGTCTGGACAGGATGGCCTACACATGGAGAGCTCTGCTG      60
                                    m  a  y  t  w  r  a  l  l

CTGCTGGCGCTGGCCTTTCTGGGCAGCAGCATGGCAGAGCGGGACTGCCGTGTGAGCAGC     120
 l  l  a  l  a  f  l  g  s  s  m  a  E  R  D  C  R  V  S  S

TTCAAAGTCAAGGAGAACTTCGACAAGAACAGGTACAGTGGCACCTGGTACGCCATGGCA     180
 F  K  V  K  E  N  F  D  K  N  R  Y  S  G  T  W  Y  A  M  A

AAAAAAGATCCTGAGGGGCTGTTTCTACAGGACAATGTGGTTGCCCAGTTCACTGTAGAT     240
 K  K  D  P  E  G  L  F  L  Q  D  N  V  V  A  Q  F  T  V  D

GAGAATGGACAGATGAGTGCCACTGCAAAGGGCAGAGTCAGACTCTTCAATAACTGGGAT     300
 E  N  G  Q  M  S  A  T  A  K  G  R  V  R  L  F  N  N  W  D

GTCTGTGCTGATATGATTGGCTCTTTCACTGACACAGAGGATCCTGCCAAGTTCAAGATG     360
 V  C  A  D  M  I  G  S  F  T  D  T  E  D  P  A  K  F  K  M

AAGTACTGGGGTGTTGCCTCTTTTCTGCAGAAAGGAAATGATGATCACTGGGTAGTGGAC     420
 K  Y  W  G  V  A  S  F  L  Q  K  G  N  D  D  H  W  V  V  D

ACAGATTACGATACATATGCTCTTCATTATTCCTGCCGTGAGCTAAATGAAGATGGCACT     480
 T  D  Y  D  T  Y  A  L  H  Y  S  C  R  E  L  N  E  D  G  T

TGTGCTGATAGCTATTCCTTTGTATTCTCCCGGGACCCCAAGGGATTGCCTCCAGAGGCA     540
 C  A  D  S  Y  S  F  V  F  S  R  D  P  K  G  L  P  P  E  A

CAGAAAATTGTTAGACAAAGGCAGATAGACCTCTGCTTGGACAGAAAATACAGAGTTATC     600
 Q  K  I  V  R  Q  R  Q  I  D  L  C  L  D  R  K  Y  R  V  I

GTTCATAATGGATTTTGCTCTTAAGGAGATCCATAAGTATGGAAGGAAACCGTGTAACAG     660
 V  H  N  G  F  C  S

TACCATGGATGTAAAGTTAATACATTGATCAGGTGCTCTTTTAAAAAGATTTTAGATGGC     720

ATCATTTAGATTTCAAATATATTTATCTGAACTGCATAGCTCATTTTATTAATAAACCAA     780

TGAAACATTTTAATAAACTTGGAGGAATTC                                   810
```

Fig. III.2. Alignment of the primary structures of human and chicken serum retinol-binding proteins (RBPs). The amino acids which differ in the two proteins are indicated as circles above the human sequence. The secondary structural elements, eight ß-strands (shaded) and the alpha-helix (boxed), are shown based on the known crystal structure of human RBP (Ref. 153).

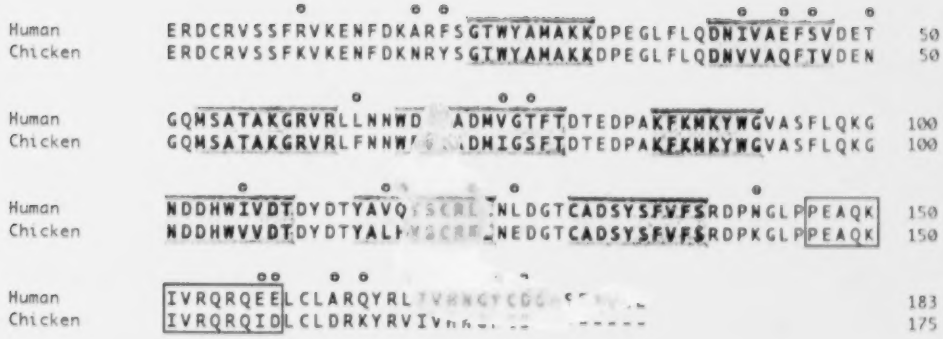

Human ERDCRVSSFRVKENFDKARFSGTWYAMAKKDPEGLFLQDNIVAEFSVDET 50
Chicken ERDCRVSSFKVKENFDKNRYSGTWYAMAKKDPEGLFLQDNVVAQFTVDEN 50
Human GQMSATAKGRVRLLNNWD ADMVGTFTDTEDPAKFKMKYWGVASFLQKG 100
Chicken GQMSATAKGRVRLFNNW DMIGSFTDTEDPAKFKMKYWGVASFLQKG 100
Human NDDHWIVDTDYDTYAVQ NLDGTCADSYSFVFSRDPNGLPPEAQK 150
Chicken NDDHWVVDTDYDTYALI NEDGTCADSYSFVFSRDPKGLPPEAQK 150
Human IVRQRQEELCLARQYRL 183
Chicken IVRQRQIDLCLDRKYRVI 175

Fig. III.3 Effects of estrogen administration on retinol-binding protein RNA levels in the rooster. Each lane of the Northern blots shown contains 10 ug of total RNA. (**A**) The ^{32}P-labelled chicken RBP cDNA was used as a probe for hepatic (lanes 1 and 3) and kidney (lanes 2 and 4) RNA. Lanes 1 and 2 contain RNA from control roosters, and lanes 3 and 4 from roosters 81 hr post-estrogenization. The upper and lower bars at the left of lane 1 indicate the position of the 28S and 18S ribosomal RNAs, respectively. A more detailed analysis of the effects of estrogen (**B**) shows the decrease in hepatic RBP RNA (top panel; probed as in (A)) at various times after one estrogen treatment (26 hr, lane 1; 81 hr, lane 2) and 49 hr (lane 3) after a second treatment which was performed 11 days after the first, relative to the unestrogenized control (lane C). In the other panels of (B), the same blot as in the top panel was reprobed with human actin (middle panel) or chicken apo VLDL II (lower panel) cDNAs in order to provide a control for RNA levels as well as a positive control for the effects of estrogenization, respectively.

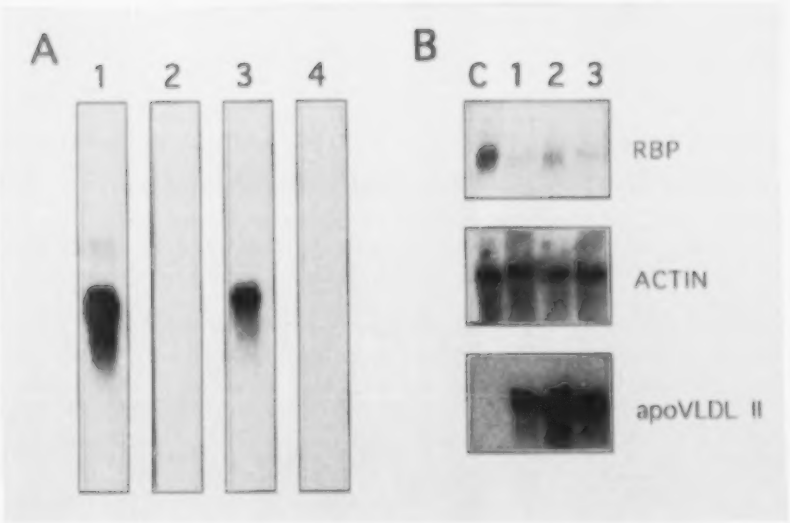

A
1
2
3
4
B
C 1 2 3
RBP
ACTIN
apoVLDL II

Fig. III.4 Effects of estrogen on rooster liver RBP gene transcription. A run-on transcription asssay was performed on isolated liver nuclei from estrogen-treated (E) or control (C) roosters, and the ^{32}P-labelled RNA transcripts were hybridized to 2 ug of a cut, denatured plasmid containing the RBP cDNA (R) or plasmid without insert (V) which had been imobilized on nitrocellulose. After an RNase digestion to remove unhybridized RNA and several washes (see Section II.S), the nitrocellulose strips were exposed to film for 9 days at -70°C.

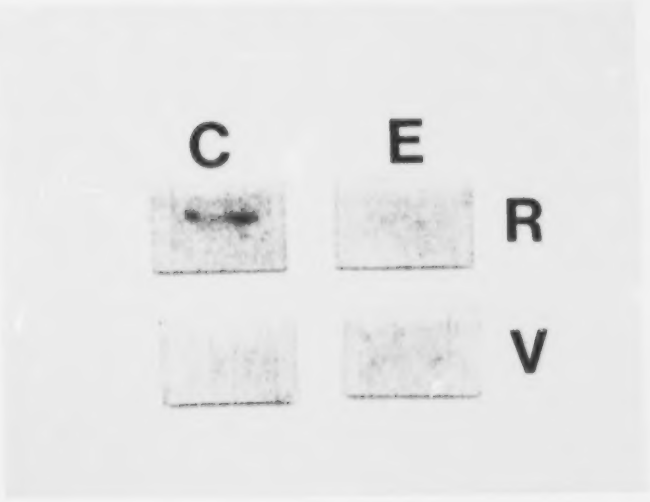

C
E
R
V

Fig. III.5 Northern blot analysis of laying hen ovarian follicle RNA for the presence of the RBP message. Total RNA (10 µg) from liver (lane 1), and 20 µg of total RNA from large (8-30 mm diameter; lane 2) and small (1-8 mm diameter; lane 3) follicles were probed with the labelled chicken RBP cDNA. The positions of the 28S and 18S ribosomal RNAs are indicated at the left. For control purposes, the bottom panel shows the fluorescence intensity of the ethidium bromide-stained 28S ribosomal RNA.

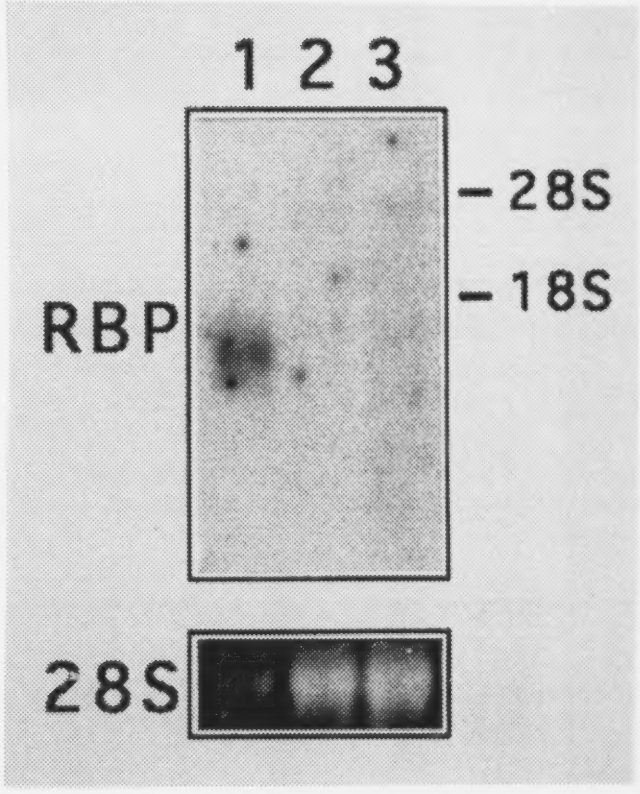

1 2 3
– 28S
– 18S
RBP
28S

Fig. III.6 Alignment of the primary structures of human and chicken TTR proteins (taken from Ref. 172). The identical amino acids are boxed. Relative to all known mammalian TTRs, the chicken protein has three extra amino acids near its N-terminus. The following residues participate in thyroid hormone binding (numbers correspond to the chicken TTR shown above): K18, E57, S120, T122.

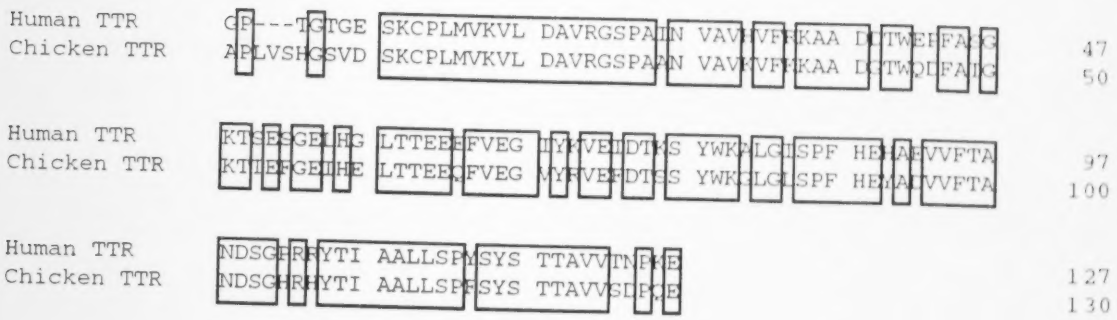
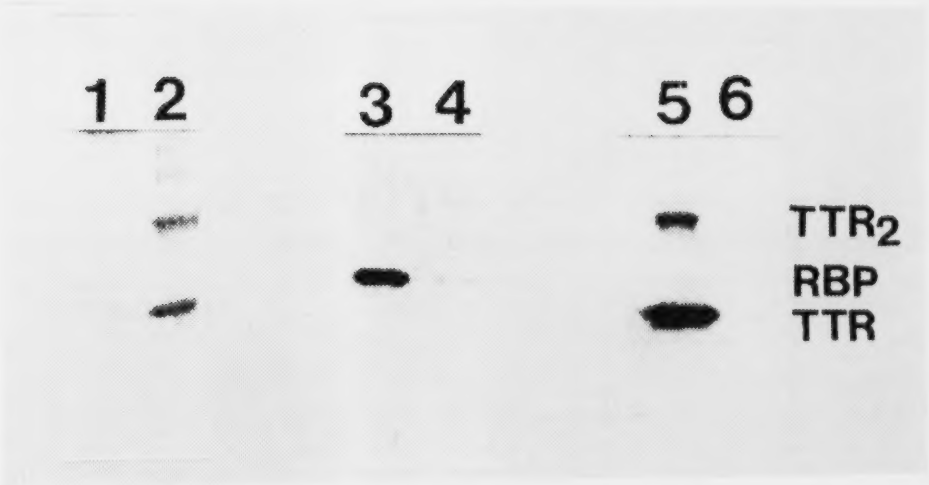

Human TTR
Chicken TTR
Human TTR
Chicken TTR
Human TTR
Chicken TTR

Fig. III.7 Specificity analysis of rabbit anti-human and anti-chicken polyclonal antibodies. Chicken RBP-TTR complex (lanes 1, 3, and 5; purified as described in Materials and Methods), and purified human TTR (lanes 2, 4, and 6; obtained from Calbiochem) were subjected to Western blotting with anti-human TTR IgG (lanes 1 and 2), anti-chicken RBP (lanes 3 and 4), and anti-chicken TTR (lanes 5 and 6). The RBP protein signal represents a molecular mass of 21 kDa; the TTR monomer and dimer (TTR_2) represent 13 kDa and 32 kDa, respectively. All samples were heated (95 degrees C for 2 min) under reducing conditions (50 mM DTT) prior to SDS-PAGE.


1 2
3 4
5 6
TTR_2
RBP
TTR

CHAPTER IV

IDENTIFICATION AND ANALYSIS OF CIRCULATORY AND OOCYTIC TTR-RBP-RETINOL COMPLEXES

In order to study the mechanisms for oocyte targeting of retinol and possibly its serum transport complex, I employed several chromatographic methods along with Western blotting and fluorescence measurements in an analysis of retinol-containing protein complexes from avian serum and oocytic yolk. A retinol-RBP-TTR complex similar to that found in mammalian serum was identified and characterized in both chicken serum and yolk.

A. GEL FILTRATION ANALYSES OF CHICKEN SERUM AND YOLK

Gel filtration chromatography monitored by retinol fluorescence (excitation at 334 nm and emission at 463 nm) indicated that in both yolk and serum a fraction with high retinol fluorescence co-eluted with the 80 kDa (conalbumin) column standard (Fig. IV.1). No other major peak of retinol fluorescence in serum or yolk could be detected between 80 kDa and the total column volume. In order to identify the retinol-containing protein(s), another gel filtration experiment (Fig. IV.2) was performed, and representative fractions of similar elution volume from yolk and serum were screened for the presence of RBP and TTR by immunoblotting with affinity-purified polyclonal antibodies against the respective chicken proteins. RBP and TTR, with apparent molecular weights on SDS-PAGE of 21,000 and 13,500, respectively, were identified in both the serum (Fig.

IV.2A) and yolk (Fig. IV.2B). These M_r values are in agreement with the reported values for RBP and the TTR monomers from other species (69).

Based on elution volumes, Fig. IV.2 shows that two populations of RBP exist in both the yolk and serum. One population has an elution volume which represents proteins in the range of 70-80 kDa in serum (Fig. IV.2A top, lane 2) and yolk (Fig. IV.2B top, lane 2), and another population of <20 and <30 kDa in both serum (Fig. IV.2A top, lane 4) and yolk (Fig. IV.2B top, lane 4), respectively. The smaller species likely represents free RBP (M_r 21 kDa). Because TTR is also present in the same fractions as the higher M_r RBP species (bottom panels of Fig. IV.2A and IV.2B, lanes 2), these fractions presumably contain the RBP-TTR complex. This finding is supported by the results of Fig. IV.1 which demonstrate the presence of retinol in a fraction representing proteins of approximately 80 kDa. The observed M_r value of 75-80,000 is in accord with previously reported values for the serum RBP-TTR complex (123) and with the sum of the M_r values for RBP (21,000) and the native TTR tetramer (55,000) in a 1:1 complex (69, 123, 125). The intense TTR signal in lane 3 of Fig. IV.2A is likely due to the presence of free TTR as well as RBP-TTR complex; because of the small size difference between native TTR (55 kDa) and RBP-TTR (75 kDa), the resolution of the free and complexed TTR populations is not as good as that for the two RBP populations.

In a separate size exclusion chromatographic analysis of yolk, the fractions representing selected size ranges were subjected to an emission spectrum fluorescence analysis (Fig. IV.3). The characteristic one-peak fluorescence spectrum of free retinol was not observed in the sample fractions. Instead, the characteristic two-peak retinol-RBP fluorescence emission spectrum (Ref. 69, and see IV.C below) was seen in the size range

of 55-85 kDa which corresponds to the RBP-TTR complex. These data support the presence of holo-RBP-TTR complex in the yolk of the chicken oocyte.

In a previous analysis of chicken yolk, a ~75 kDa retinol-protein complex, likely involving RBP, was detected in aqueous extracts obtained by subjecting yolk to dialysis, ion exchange chromatography and ammonium sulfate precipitation (175); no other retinol containing complexes were detected in the unextracted yolk pellets (175). The 2-day-long yolk preparation procedure in that study, however, increased the possibility of a non-physiological association of proteins in the aqueous phases. To decrease the possibility of significant in vitro association, I employed a novel, rapid freeze-thaw method for obtaining the aqueous yolk extract used for my experiments (Figs. IV.1-3). Thus, the data from gel filtration chromatography which I show indicate that a physiological retinol-RBP-TTR complex exists in chicken serum and yolk.

B. IMMUNOAFFINITY CHROMATOGRAPHY AND PURIFICATION OF YOLK AND SERUM TTR-RBP

A novel procedure was developed to purify the retinol transport complex and its components by using an immunoaffinity matrix of immobilized anti-chicken TTR IgG. This antibody to TTR does not recognize RBP on Western blots (Fig. IV.2 and Fig. III.7). The antibody column is expected to interact only with free TTR or RBP-TTR complexes which may be present in serum and yolk. (For the purpose of obtaining

pure TTR and RBP preparations from a mixture of free and RBP-complexed TTR, a low ionic strength gel filtration method was employed as described in Section II.E.1).

The results of the specific immunoaffinity step of the purification procedure are shown in Fig. IV.4; they provide evidence for the existence of the RBP-TTR complex in yolk. The Coomassie-blue stained protein eluates, shown in lanes 5 and 6 after SDS-PAGE, correspond to RBP (21 kDa) and TTR (13.5 kDa monomer). Immunoblot analysis (e.g., shown in Fig. III.7) of these anti-TTR column eluates confirms the identity of the RBP and TTR protein bands. Thus, the presence of the RBP-TTR complex in chicken serum and yolk is also demonstrated using an immunoaffinity chromatographic method.

C. FLUORESCENCE PROPERTIES OF PURIFIED RBP AND RBP-TTR COMPLEXES FROM YOLK AND SERUM

To confirm that yolk RBP (purified from the RBP-TTR complex as described in "Materials and Methods") contains retinol, its fluorescence excitation spectrum was compared with that of serum RBP, known to be saturated with retinol (69). The spectrum (Fig. IV.5) shows that both RBPs contain the two characteristic excitation peaks when fluorescence emission is monitored at 463 nm. The peak at ~330 nm represents the direct excitation of retinol, while that at 280 nm represents the indirect excitation of retinol by energy transfer from aromatic amino acids (trp) of the protein (69). This spectrum of retinol-RBP differs from that observed upon

fluorescence analysis of free retinol (cf. Fig. IV.3), or from that of other retinol-protein complexes (176, 177). The same two-peak emission spectrum as that shown in Fig. IV.5 was observed for the pure serum and yolk TTR-RBP complexes eluted from the anti-TTR immunoaffinity column and suggests that the RBP structure is not greatly altered upon its interaction with TTR, i.e., the position of the retinol does not change significantly relative to the amino acids that transfer energy to it. The lack of significant changes in the structure of RBP when it is complexed to TTR has also been suggested based on antibody competition experiments (178).

D. DIFFERENCES BETWEEN SERUM AND YOLK RBP, TTR AND RBP-TTR COMPLEX

For both serum and yolk, the chromatography data show the presence of free RBP and TTR in addition to the complex. From Western blots of the gel filtration fractions such as those in Fig. IV.2, it is not possible to make conclusions about the relative abundance of free and complexed RBP or TTR in yolk and serum because the intensity of the respective signals is only relative to the total protein present in that particular size range. However, the affinity chromatography method described in Section IV.B revealed that the yolk (Fig. IV.4, lane 6) yields a lower RBP/TTR ratio than the serum (Fig. IV.4, lane 5). This was confirmed in several subsequent chromatrographic runs. It may be that either the TTR or the RBP moiety, or both, are modified in yolk and that this modification decreases the stability of the complex. Limited

proteolysis of other serum proteins following their uptake by chicken oocytes, e.g. of apolipoprotein B, vitellogenin, and riboflavin-binding protein, has been detected (179-181). It is also possible that oocytes accumulate free TTR in addition to the RBP-TTR complex. If free TTR is preferentially accumulated in yolk, this may result in a relative RBP deficit. Uncomplexed TTR does exist in the serum of man (69), rat (69), and chicken (Fig. IV.2A).

A minor difference was observed in the electrophoretic mobility of a small fraction of dimeric TTR in yolk vs. serum upon immunoblotting with an anti-chicken TTR antibody that had been immunopurified on immobilized TTR monomer (Fig. IV.6). However, the observed difference between the yolk and serum TTR dimeric structures may also reflect a difference in the amount of TTR that has bound thyroid hormone, or other small, minor components that have been found associated with human TTR (182). The nature and significance of this minor difference in the mobility of the dimer remains unknown, but it was consistently observed. The relative levels of the normal and altered dimeric TTR structure in the yolk often varied in different preparations. Incubation of yolk or serum at 37°C for 7 hours, however, did not alter the relative levels of the normal and modified dimeric species observed. The different dimeric species are not apparent upon SDS-PAGE analysis and Coomassie staining of purified yolk and serum TTR (Fig. IV.7); although this may due to weak staining of dimeric structures. At this point, it remains unknown whether this minor TTR modification is one cause of the lower RBP/TTR ratio in yolk relative to serum. I attempted to determine if the various TTR species, after SDS-PAGE and transfer to nitrocellulose, could interact with RBP. Under the various conditions that I used in these ligand blot experiments, however,

the demonstration of RBP interaction with modified or normal TTR was not successful.

In the case of RBP, no difference in the electrophoretic mobility of yolk vs. serum could be detected upon Western blotting or SDS-PAGE followed by Coomassie blue staining using 4.5-18% or 10-20% acrylamide gradients (see Fig. IV.8 as an example). Fig. IV.8, as mentioned previously, reveals that both yolk and serum RBP are highly compacted in their structure by disulfide bonds. Yolk RBP was fully competent to bind TTR as shown by the previous demonstrations of the RBP-TTR complex in yolk. In addition, precipitation of chicken yolk RBP with human TTR-Sepharose or anti-chicken TTR IgG-Sepharose (Fig. IV.9) also revealed the capacity of yolk RBP to interact with TTR. A minor difference between pure yolk and serum retinol-RBP was detected by comparing the fluorescence excitation peaks, i.e., by measuring the height of the 280 nm and 334 nm excitation peaks (such as the ones shown in Fig. IV.5) that represent the 463 nm emission. This slight difference may reflect a small change in RBP structure, assuming that the yolk and serum RBP retinol ligands are not differentially modified. A small change in the RBP structure may change the distance, for example, between retinol and one of its interacting amino acids and thus change the efficiency of the energy transfer.

E. THE RBP-TTR COMPLEX

This section represents a brief speculation on the residues involved in the RBP-TTR interaction. The suggestions in this section should be

tested with appropriate structural models; they can also be directly tested by site-directed mutagenesis. The interaction sites between these two proteins have been speculated upon for many years; however, no specific surfaces of interaction have yet been defined. Upon analysis of the known data on RBP-TTR interaction and a homology comparison of the novel chicken RBP and TTR structures presented previously (Figs. III.1 and 6), I propose that RBP residues A84, K85, F86, K87, M88, K89, Y90, W91 interact with TTR residues L20, D21, F76, D77, T78, D95, V96, V97, respectively, for the following reasons:

(i) These residues lie close to each other on their respective proteins and some of the polar and charged side chains are located on the surface as determined by X-ray crystallography (153, 170).

(ii) The pKa's of K and D/E may be an important factor in the observed dissociation of the complex at pH>9 and pH<5.

(iii) Dissociation of the complex at low ionic strength may reflect reduced hydrophobic interactions between these residues, e.g., A, L, F, Y, V, W.

(iv) The interaction would involve alternating hydrophobic-hydrophobic and ionic interactions, e.g., A84/L20, K85/D21, F86/L76, etc.

(v) Some of these RBP residues may be iodinated (Y90) or biotinylated (K90). This may explain the interference of TTR in such labellings of RBP.

(vi) Both of these RBP and TTR regions are highly conserved in the known respective proteins and are not very antigenic (178); RBPs and TTRs from different species can cross interact (Ref. 125 and Fig. IV.9).

(vii) The RBP region is absent in purpurin, a protein which is highly homologous to RBP but does not interact with TTR (183).

(viii) The RBP region is partly on a loop close to the retinol-binding pocket and it has been found that substitution of bulky residues at the exposed retinol OH group interfere (see Fig. I.7) with TTR interaction (184).

Fig. IV.1 Serum and yolk contain an ~80 kDa retinol fluorescent species. Whole laying hen serum (filled circles) and aqueous yolk extract (filled triangles) were fractionated by gel filtration chromatography (see Section II.G), and the 463 nm fluorescence emission of the fractions was measured with an excitation wavelength of 334 nm. The positions of elution of size standards are indicated at the top (open triangles): 33 kDa, riboflavin-binding protein; 80 kDa, conalbumin; 170 kDa, chicken IgG. V_O is the void volume.

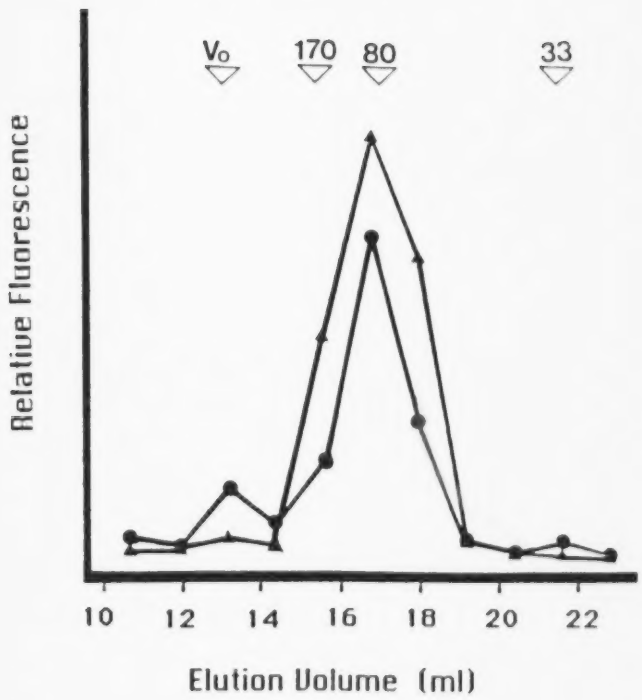

Vo
170
80
33
Relative Fluorescence
10
12
14
16
18
20
22
Elution Volume (ml)

Fig. IV.2 Presence of RBP and TTR in chicken oocytes. Laying hen serum (900 µl) and aqueous yolk extract (900 µl) were fractionated by gel filtration on Sephadex G-100. Representative fractions of the serum (A) and yolk (B) were subjected to Western blotting with affinity-purified antibodies against RBP (top panels) and TTR (bottom panels), respectively. SDS-PAGE molecular mass standards (kDa) are indicated at the left of each figure. The median molecular mass of the proteins in each fraction, obtained from a standard curve of K_{av} vs. log (M_r of the column standards), is indicated in the boxes between top and bottom panels. The following proteins were used to standardize the gel filtration column: rabbit IgG (150 kDa), conalbumin (80 kDa); BSA (69 kDa), and riboflavin-binding protein (33 kDa). In each lane, 60 µg of yolk protein or 30 µg of serum protein was loaded.

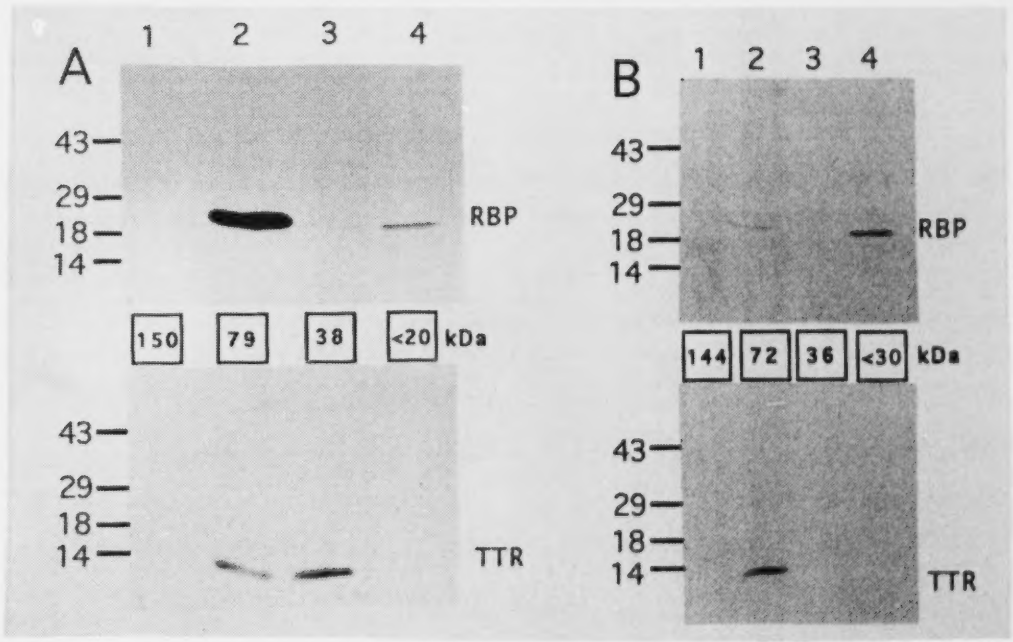

A
1 2 3 4
43
29
18
14
RBP
150 79 38 <20 kDa
43
29
18
14
TTR
B
1 2 3 4
43
29
18
14
RBP
144 72 36 <30 kDa
43
29
18
14
TTR

Fig. IV.3 Fluorescence analysis of representative gel filtration fractions of yolk or a pure retinol standard (Sigma). Excitation peaks which yield emission at 463 nm are shown. Note that the relative fluorescence intensity (I) of the samples in the upper two panels is approximately five-fold lower than in the other two samples. Molecular mass ranges of the fractions are shown in the upper right corner of the panels (kDa).

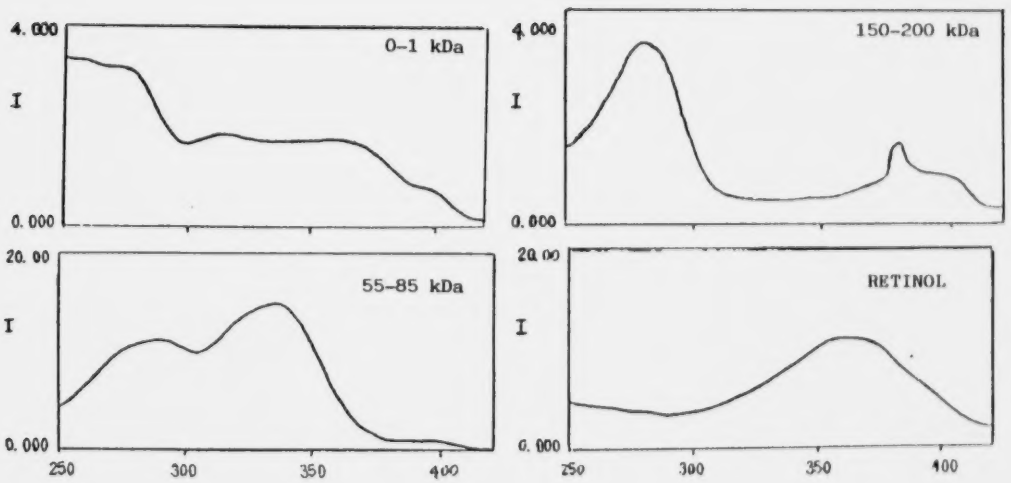

4.000
I
0.000
0-1 kDa
4.000
I
0.000
150-200 kDa
20.00
I
0.000
55-85 kDa
20.00
I
0.000
RETINOL
250
300
350
400
250
300
350
400

Fig. IV.4 Purification of yolk and serum retinol protein complexes. The results from the anti-TTR immunoaffinity step of the purification procedure are shown as Coomassie Blue-stained 4.5-18 % gradient SDS polyacrylamide gel. The starting material is either laying hen serum (lane 1) or aqueous yolk extract (lane 2), both after anion exchange chromatography as described in Section II.E. The respective unbound fractions (flow-through) from the immunoaffinity column are shown in lanes 3 and 4. The bound fractions are shown in lanes 5 and 6 for the serum and yolk samples, respectively. Molecular mass standards (kDa) are indicated. Lanes 1-4 contain 60 μg of protein each, and lanes 5 and 6 contain 2 μg each.

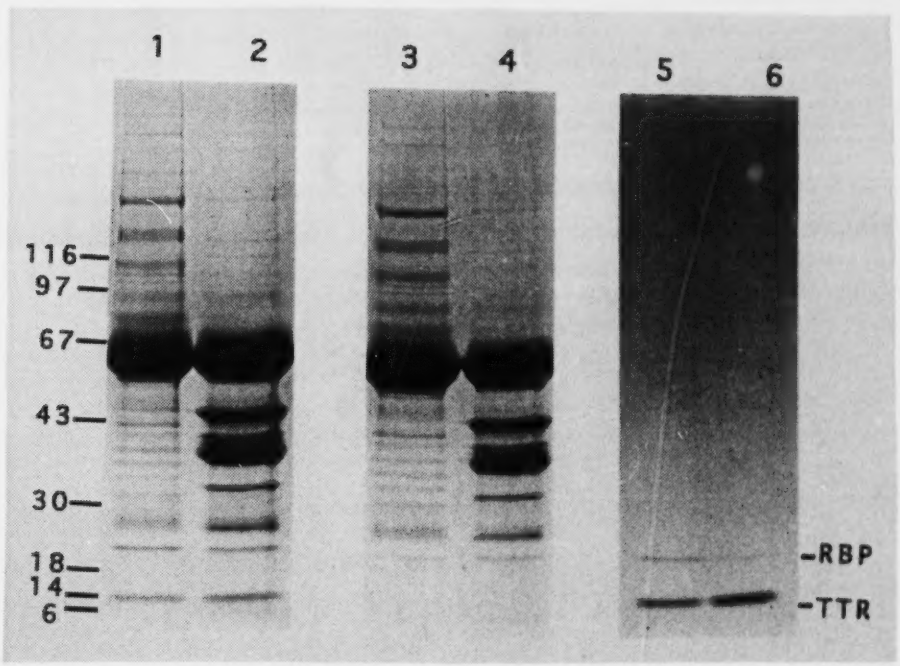

1
2
3
4
5
6
116
97
67
43
30
18
14
6
-RBP
-TTR

Fig. IV.5 Fluorescence spectra of pure yolk and serum RBP. Fluorescence excitation spectra of the samples (~20 μg) dissolved in PBS were obtained with on a Hitachi F200 Fluorescence Spectrophotometer using a fixed emission wavelength of 463 nm. 1: serum RBP; 2: yolk RBP; and 3: buffer control (PBS).

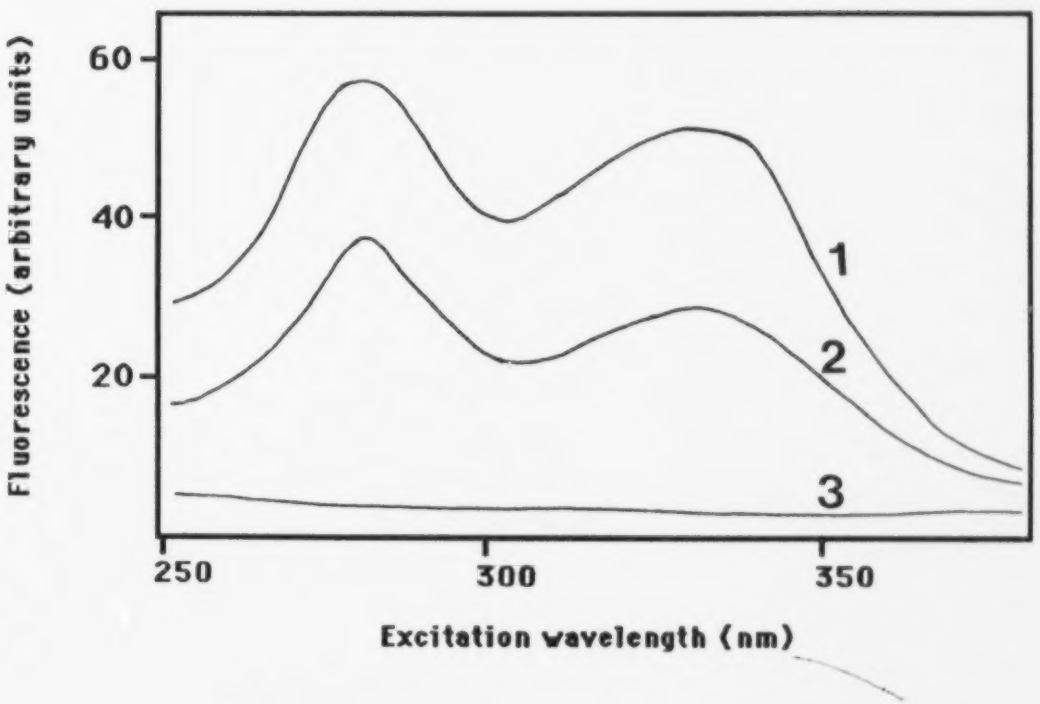

Fluorescence (arbitrary units)
60
40
20
1
2
3
250
300
350
Excitation wavelength (nm)

Fig. IV.6 Comparison of yolk and serum chicken TTRs by Western blotting. (A) Purified yolk (2µg; lane 1) and serum (2µg; lane 2) TTR were heated (95°C for ~3 min), subjected to SDS-PAGE under reducing conditions and electrotransferred to nitrocellulose. An anti-chicken TTR polyclonal antibody which had been purified on immobilized TTR monomer was used to probe the blots. (B) Whole laying hen serum (86 µg; lane 1) and a crude yolk aqueous extract (130 µg; lane 2) were treated and blotted as in "(A)". The TTR monomer protein bands (13 kDa) are visible near the bottom of the blots. Higher order 33 kDa and 31 kDa TTR structures (likely dimers, see text) are marked by open and filled arrowheads.

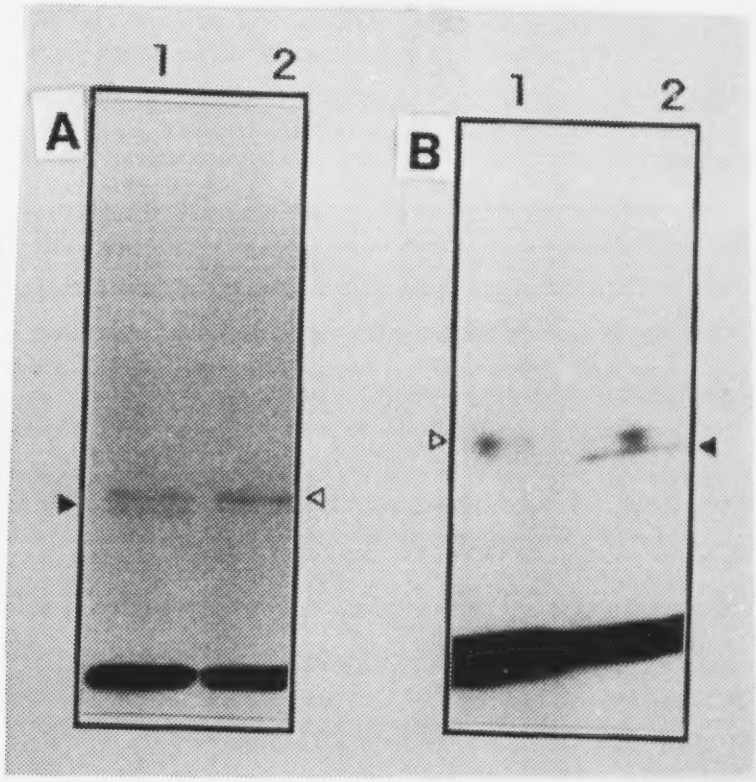

1
2
A
1
2
B

Fig. IV.7 Comparison of yolk and serum TTRs by SDS-PAGE and Coomassie-blue staining. Pure yolk TTR (2 µg; lanes 1 and 4) and pure serum TTR (2 µg; lanes 2 and 3) samples containing 150 mM SDS and 90 mM DTT were either heated for 10 min at 90°C (lane 3 and 4) or kept for 20 min at room temperature (lanes 1 and 2) before electrophoresis. The molecular masses of the dimeric (lanes 1 and 2) and monomeric (lanes 3 and 4) TTRs observed are ~32 kDa and ~13 kDa, respectively.

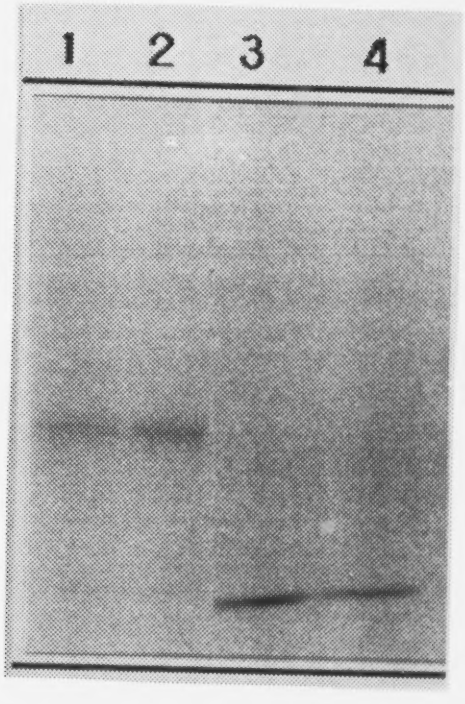

1
2
3
4

Fig. IV.8 Comparison of the electrophoretic mobilities of yolk and serum RBP by SDS-PAGE and Coomassie-blue staining. Purified yolk RBP (lanes 1 and 4) and serum RBP (lanes 2 and 3) were heated and either reduced (90 mM DTT; lanes 3 and 4) or not reduced (lanes 1 and 2) before electrophoresis. Molecular mass standards representing (from top to bottom) 69 kDa, 45 kDa, 30 kDa, 18 kDa and 14 kDa are shown to the right of lane 4.

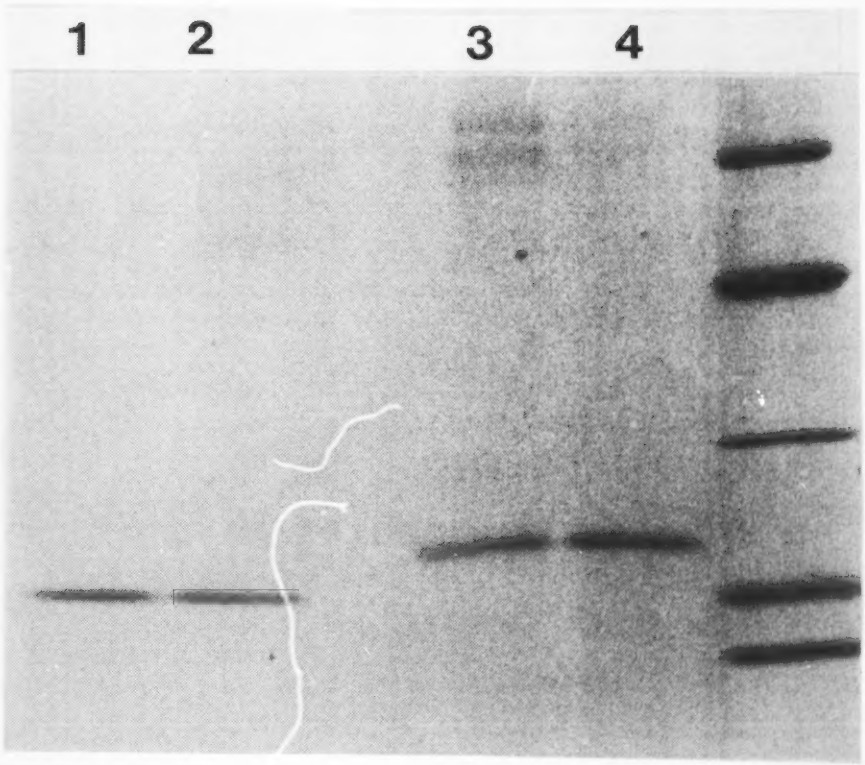
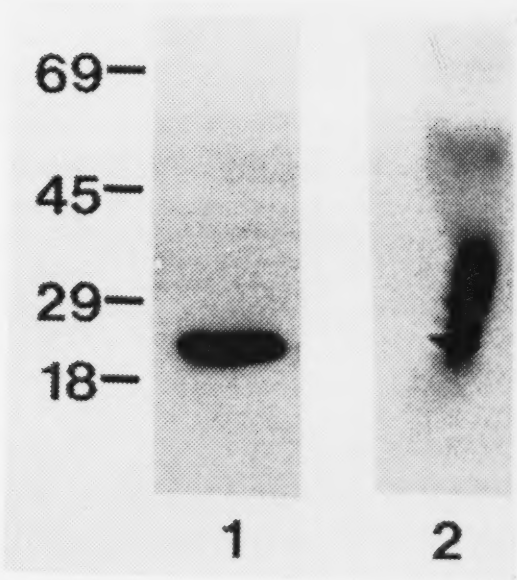

1
2
3
4

Fig. IV. 9 Demonstration of interaction between chicken yolk RBP and human serum TTR. A yolk aqueous extract was precipitated with either human TTR sepharose (lane 1) or, as a control, with anti-chicken TTR IgG and protein A-Sepharose (lane 2). The precipitates were washed extensively with PBS and analysed by Western blotting with anti-RBP IgG. The IgG heavy chain signal was excised to enable clearer demonstration of the RBP signal. Control immunoprecipitations of yolk with antibodies against other proteins followed by protein A sepharose did not result in RBP precipitation.


69—
45—
29—
18—
1
2

CHAPTER V

OOCYTIC ENDOCYTOSIS OF RBP AND TTR AND ANALYSIS OF THEIR YOLK DEPOSITION

The data of the previous chapters suggest that the serum retinol-RBP-TTR complex is taken up by the oocyte and deposited in the yolk. No evidence for the synthesis of RBP in the ovarian follicle could be obtained in Northern blots and PCR analysis. To provide more direct evidence for the uptake of serum RBP and TTR by the oocyte, I have (i) performed *in vivo* experiments to show the transfer of labelled proteins from the serum into the yolk, (ii) prepared endocytic clathrin-coated vesicles from the oocytes to show the presence of RBP and TTR, and (iii) analyzed oocyte yolk by immunoelectron microscopy to confirm the deposition of the proteins in the yolk mass and show their distribution relative to various known yolk structures and other yolk precursors.

A. UPTAKE OF LABELLED SERUM TTR BY THE OOCYTE

To determine if circulatory TTR (and RBP, see below) is the precursor of TTR found in the growing oocytes, whole laying hen serum was biotinylated and reinjected into the hen after removal of free biotinylation reagent. This biotin-based labelling protocol was selected instead of radioiodination because of its greater sensitivity when combined with ECL detection, and in order to avoid performing *in vivo* experiments with large quantities of radioactivity. The biotinylation reaction was highly efficient as determined by probing control and biotinylated serum proteins, after SDS-PAGE and transfer to nitrocellulose, with streptavidin-HRP. Subsequent chromatography of yolk from oocytes of the injected hen

on an anti-TTR sepharose column and biotin-specific blotting (with streptavidin-HRP) of the immunoaffinity column eluate revealed the presence of serum biotin-TTR in the oocyte (Fig. V.1A, lane 1 and Fig. V.1B, lane 3). The control yolk from an uninjected hen, as expected, was only positive for the TTR analysis with the antibody (Fig. V.1B, lane 4) but not for biotin-TTR (Fig. V.1A, lane 2). Thus, these data show that circulatory TTR can be endocytosed by the chicken oocytes in vivo.

Approximately half of the TTR in the circulation of vertebrates is present complexed to RBP (14, 69 and Refs. therein) and TTR-RBP complexes can be isolated from yolk (Chapter IV); however, the biotinylation technique described here for TTR cannot be applied to analyze the oocytic uptake of the RBP-TTR complexes because complexed RBP is not readily labelled by biotinylation (nor by iodination; as mentioned in Section IV.E). It is likely that RBP, complexed with biotin-TTR in the serum, also entered the oocyte because RBP was detected upon immunoblotting of biotin-TTR samples such as those in Fig. V.1 with an anti-RBP polyclonal antibody. Although it is not possible to determine what fraction of the yolk TTR sample from the biotinylated serum-injected hen represents biotin-TTR relative to normal TTR, the presence of RBP in this fraction suggests that some biotin-TTR-RBP complex entered the oocyte; the alternative, that biotin-TTR associated in the yolk with free RBP, is also possible; but it may be less likely that this complex can form in the very lipid-rich environment of the yolk. In any case, if biotin-TTR can associate with RBP in the yolk it should also associate with RBP in the serum.

One additional important result from the above biotin-serum experiment is that no biotinylated components were detected in the egg-

white upon analysis (>200 ug of protein/gel lane) with streptaviding-HRP. This represents the first direct support for the hypothesis that the egg-white is composed only of oviduct products and there is no deposition of even small amounts of serum components.

B. OOCYTIC RBP AND TTR: STAGE SPECIFIC ACCUMULATION DURING YOLK FORMATION AND TRANSPORT IN CLATHRIN-COATED VESICLES

Western blots of yolk provided biochemical evidence for the presence of RBP and TTR (Chapter IV). Western blotting of the yolk at various stages of oocyte growth (follicular sizes), showed that both RBP and TTR were more abundant relative to total yolk protein at early stages, <5 mm diameter (Fig. V.2). The similarity of the RBP and TTR accumulation patterns is consistent with uptake of the RBP-TTR complex (and possibly some free TTR, see Fig. V.2 lane 3) by the oocyte. Although, these data do not exclude the possibility that differential degradation kinetics of RBP and TTR account for the observed accumulation pattern. The fact that the relative amount of yolk albumin (Fig. V.2 lower panel, and Fig. V. 3 top panel) does not show growth-stage dependent changes relative to total protein mass, may indicate that in oocytes, as in many somatic cells, albumin enters by a non-specific, fluid phase process (24, 86, 185-187). As mentioned in Section II.I, the unpurified anti-RBP polyclonal antibody recognizes serum albumin. The specificity of albumin recognition is shown in Fig. V.3. The immunopurified anti-RBP antibody does not

recognize albumin (Fig. V.4A). Thus, the albumin and RBP reactivies in the unpurified antibody represent two distinct clonal populations. In Fig. V.4B the albumin:RBP ratio is shown to be much higher in serum than in yolk. Although, this figure does not strictly rule out that modification of yolk albumin is responsible for the weaker signal. The albumin:TTR ratio is also likely to be much higher in serum than yolk based on the TTR signal in Fig. V.2, and the relative intensities the TTR and albumin signals observed in serum and yolk after incubation with ^{125}I-thyroxine, a known ligand of both proteins, and SDS-PAGE. Overall, these data suggest that the accumulation mechanism of RBP and TTR is different from that of chicken serum albumin.

In support of the hypothesis that RBP and TTR are endocytosed by the chicken oocyte, possibly by a receptor-mediated mechanism, RBP and TTR were found in oocytic clathrin-coated vesicles (CCVs) (Fig. V.5B). These CCV preparations have been characterized by electron microscopy (Refs. 53, 54) to show that they are virtually free of other, non clathrin-coated vesicles. As additional controls, the CCVs are characterized by SDS-PAGE to reveal the expected (53, 146), abundant clathrin heavy chain (Fig. V.5A, lane 1); and two previously characterized (54-56) oocytic lipoprotein receptors (95 kDa, 380 kDa; see Fig. VI.1, lane 3) are demonstrated in the CCVs by Western blotting (Fig. 5A, lane 2). The oocytes of the chicken and other oviparous species are cells with unusually high levels of endocytic activity as determined by their growth rate and density of clathrin-coated pits and CCVs. The data presented above are consistent with an oocytic retinol uptake mechanism that involves endocytosis of the intact RBP-TTR complex, and perhaps also some of free TTR and RBP, from the serum compartment.

C. ULTRASTRUCTURAL ANALYSIS OF YOLK FOR RBP AND TTR

In order to localize the exogeneously derived RBP in the oocytes at the ultrastructural level, immuno-electron microscopic analyses of yolk were performed (Fig. V.6). This approach was based on previous findings, from biochemical analyses of yolk, that RBP is indeed present in the chicken oocyte (Chapter IV). With an affinity-purified polyclonal antibody to chicken RBP followed by protein A conjugated to colloidal gold, RBP was demonstrated throughout the electron-lucent phase of the yolk mass but not in the electron-dense bodies. The dense bodies appear as dark, round shapes in Fig. V.6. The electron-lucent yolk subcompartment is known to contain VLDL, a serum lipoprotein that is transported via receptor-mediated endocytosis (95 kDa receptor, see Section I.B) from the plasma into the growing oocytes (53, 54). This electron-lucent phase is believed to correspond to an intermediary endosomal compartment (53, 54, Section I.B). Analysis of chicken oocyte yolk by immuno-electron microscopy also showed the presence of TTR in the yolk mass (Fig. V.7). TTR, like RBP, was localized mainly in the electron-lucent phase.

As mentioned previously, certain yolk precursors whose endocytosis by the oocyte is not believed to be receptor-mediated, e.g., ferritin (48, 49) and high density lipoprotein (HDL, Appendix B), are localized preferentially in the electron-dense phase. In the case of HDL,

two of its apolipoprotein components, apo AI and apo D, have been detected in oocytic CCVs (Appendix B). Even without definitive knowledge of the oocytic HDL uptake mechanism(s), it is possible to conclude that at least one different process exists in the endocytosis and deposition of HDL vs. RBP (or TTR) which leads to their segregation into different yolk subfractions after initially travelling together in the CCVs.

Fig. V.1 Detection of intravenously administered, biotinylated chicken serum transthyretin (TTR) in the yolk. Whole laying hen serum was biotinylated and then reinjected into a laying hen. Subsequently, oocytic yolk from the injected (lanes 1 and 3) or a control hen (lanes 2 and 4) was subjected to anti-TTR immunoaffinity chromatography (see Section II and Fig. IV.4). The column eluates were analysed for biotinylated proteins with streptavidin-HRP (**A**) and for TTR with anti-TTR IgG (**B**); detection was performed by the ECL method. The positions of migration of the two expected TTR species (monomer, 13 kDa; dimer, 30 kDa) are indicated by arrowheads. The two larger size protein bands observed in lane 1 likely represent biotinylated proteins that interact non-specifically with the IgG-Sepharose immunoaffinity matrix.

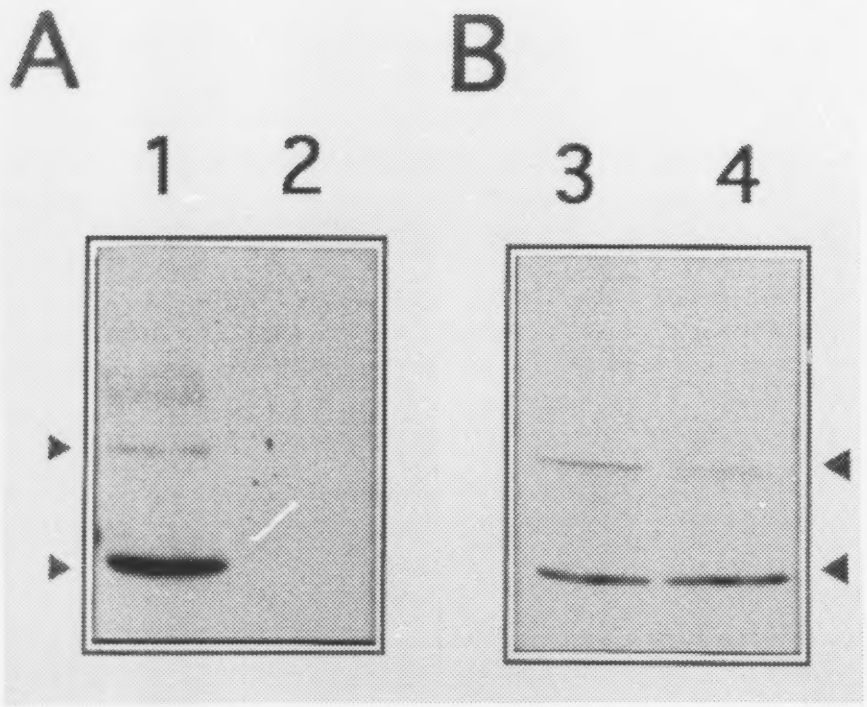

A
B
1
2
3
4

Fig. V.2 Relative levels of RBP and TTR at different stages of oocyte growth. Follicles were pulled apart with tweezers and the oocytic yolk was collected and centrifuged for 2 minutes at full speed in microfuge. Western blotting with antibodies against TTR and RBP (upper panel) and serum albumin (lower panel) was performed on 70 µg of protein from each supernate. The diameters of the processed follicles are as follows: lane 1, 2 mm; lane 2, 5 mm; lane 3, 8 mm; lane 4, 14 mm. The molecular mass standards (kDa) are shown at the left of each panel.

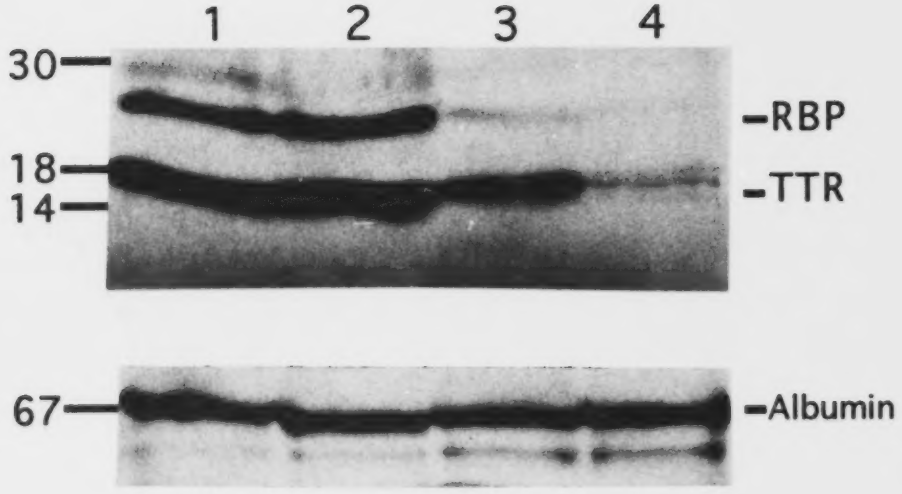

1
2
3
4
30
18
14
67
RBP
TTR
Albumin

Fig. V. 3 Characterization of the polyclonal antibody against chicken serum albumin and analysis of albumin levels in oocytes at different stages of growth. In the top panel, 100 µg yolk from oocytes of different diameters (lane1, 4 mm; lane 2, 8 mm;lane 3, 12 mm) and 5µg of purified chicken serum albumin (lane 4; obtained from Sigma) were subjected to Western blotting with the rabbit anti-chicken albumin antibody. In the bottom panel, the same samples were treated as above, except that the Western blot was performed in the presence of 100-fold molar excess of pure chicken Albumin. Bars to the right of lane 4 represent the electrophoretic mobility of molecular mass standards (from top to bottom) 95 kDa, 69 kDa, 45 kDa.

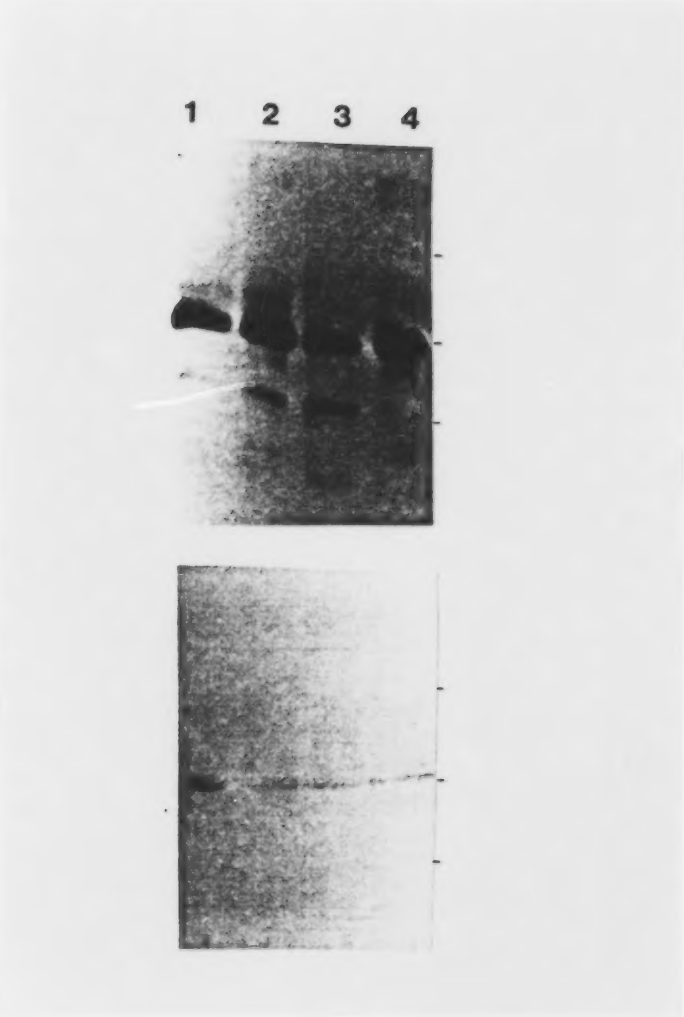

1
2
3
4

Fig. V.4 Specificities of the polyclonal anti-chicken RBP/albumin antibody and analysis of the RBP:albumin ratios in chicken yolk and serum. (A) Whole laying hen serum was subjected to Western blotting with unpurified anti-RBP/albumin IgG (lane 1) or the same antibody after affinity-purification on immobilized RBP (lane 2). (B) Whole laying hen serum (lane 1), rooster serum (lane 2), or yolk (lane 3) were analyzed by Western blotting with the unpurified anti-RBP/albumin IgG. The bars to the right of lane 3 indicate the position of albumin (SA; 69 kDa) and RBP (21 kDa).

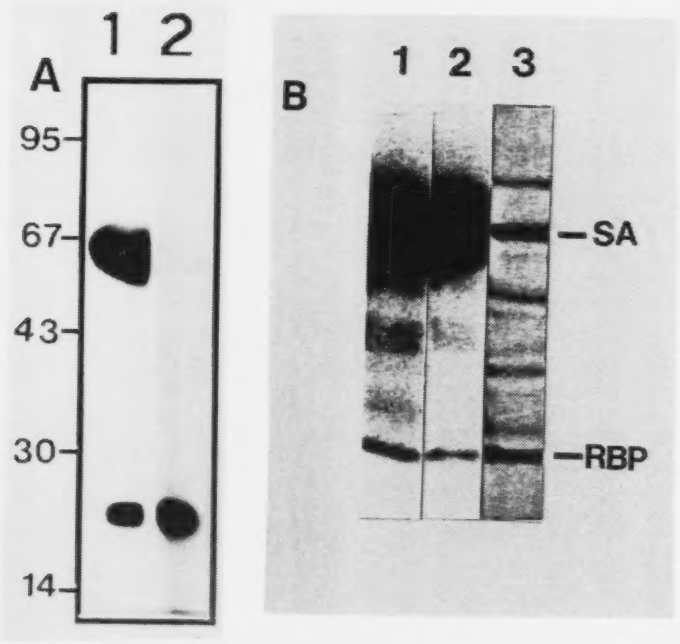

A
1 2
95
67
43
30
14
B
1 2 3
SA
RBP

Fig. V.5 RBP and TTR are present in oocytic clathrin-coated vesicles. **A**. Characterization of the coated vesicle preparation. Coomassie-blue stained gel of CV components (15 µg protein) after SDS-PAGE shows the prominent clathrin heavy chain protein band. **B**. Western blots of CV components (40 µg) with ligand-purified antibodies against TTR (lane 1) and RBP (lane 3). Lane 2 is a Western blot of CV components (30 µg) which shows the two (95 kDa and 380 kDa LRP) oocytic plasma membrane receptors involved in lipoprotein endocytosis (Section I.B).

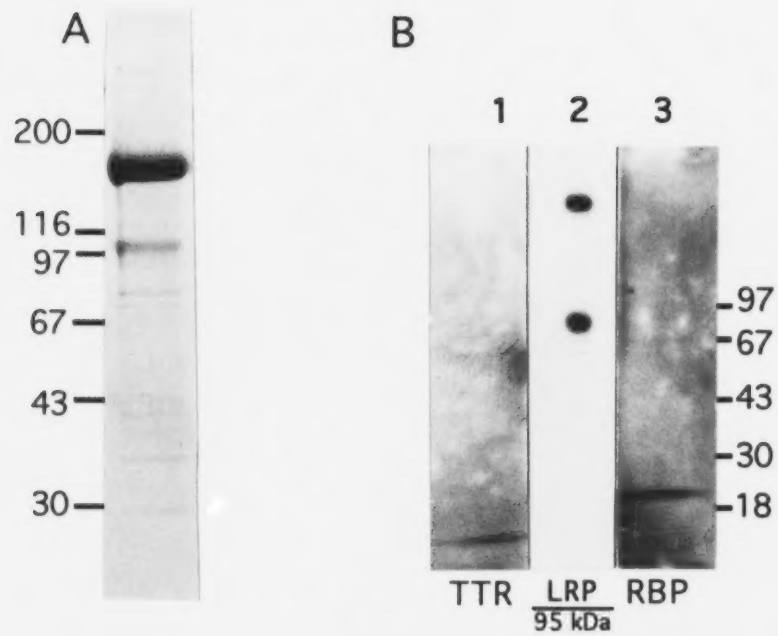

A
200
116
97
67
43
30
B
1
2
3
97
67
43
30
18
TTR
LRP
95 kDa
RBP

Fig. V.6 Ultrastructural analysis of chicken oocyte yolk for retinol-binding protein. A yolk section from an oocyte at the 5 mm (diameter) stage of growth was analysed by immuno-electron microscopy with affinity-purified antibody against chicken serum RBP (see Fig. V. 4A) as described in Section II.M. The antigen is shown to be localized in the electron-lucent phase (75 ±15 grains/μm^2) and almost completely excluded from the electron-dense granules (4 ±3 grains/μm^2). Control sections incubated with preimmune IgG showed randomly distributed grains whose abundance was <2% of that seen in this figure. The scale bar represents 1 μm.

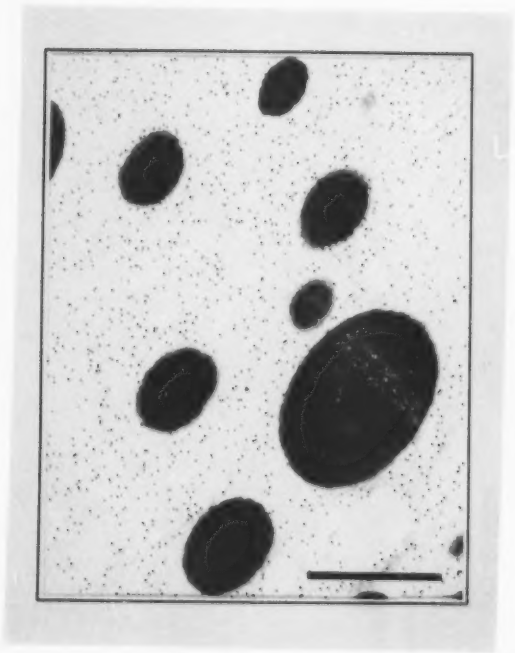
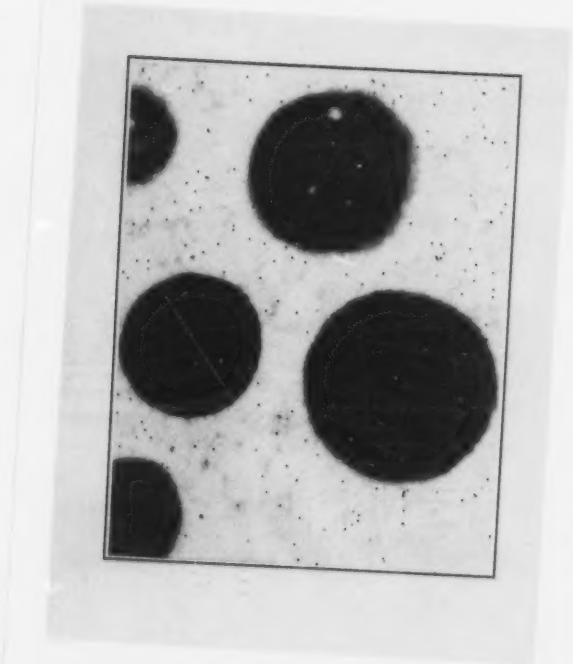


Fig. V.7 Ultrastructural localization of transthyretin (TTR) in the yolk of a chicken oocyte (diameter, 1 cm). A yolk section was analysed by immuno-electron microscopy with affinity-purified antibody against chicken serum TTR followed by protein A-conjugated gold (see Section II.M). The antigen is shown to be localized in the electron-lucent phase (19 ±5 grains/μm^2) and almost completely excluded from the electron-dense granules (1.6 ±2 grains/μm^2). Control sections incubated with non-immune IgG showed randomly distributed grains whose abundance was <5% of that seen in this figure. (Magnification, 28, 700X).



CHAPTER VI

IDENTIFICATION AND PARTIAL CHARACTERIZATION OF AN OOCYTE MEMBRANE TTR RECEPTOR

As suggested by the results of the previous chapter, oocytic uptake of the serum RBP-TTR complex likely involves a specific receptor-ligand interaction at the cell surface. To test this possibility, attempts were made to identify oocytic receptors for RBP or TTR using different ligand blot techniques and chemical crosslinking methods. Initial studies using RBP as the ligand proved unsuccessful in the identification of any oocyte membrane RBP receptor. With TTR as the ligand, however, the presence of a specific oocytic TTR receptor was consistently detected. These and other results presented below suggest that oocytic uptake of TTR and the RBP-TTR complex are mediated by a membrane TTR-binding component.

A. THE OOCYTE MEMBRANE 115 kDa TTR RECEPTOR

With evidence from other studies (13, 52-54, 56, 186) that oocytic uptake of certain serum components in oviparous organisms is a specific, receptor-mediated process, oocyte membrane proteins were tested for the presence of TTR-binding components by ligand blotting (Figs. VI.1 and 2) and chemical crosslinking (Fig. VI.3) procedures. The ligand blots with ^{125}I-TTR revealed a binding component with a relative molecular mass of approximately 115 kDa (Fig. VI.1, lane 1). The binding of ^{125}I-TTR to the 115 kDa protein could be competed by unlabelled TTR (Fig. VI.1, lane 2) but not by the bovine serum albumin present in the ligand blot buffer. This component was distinct from the abundant and well characterized 95 kDa/380 kDa oocytic lipoprotein receptors (56) (Fig. VI.1,

lane 3). Membranes from cultured chicken granulosa cells (somatic cells which enclose the oocyte in the ovarian follicle) and chicken embryo fibroblasts were also tested by the same ligand blot procedures, but no TTR-binding component could be detected (Fig. VI.1, lanes 4 and 5). By ligand blotting with ^{125}I-LDL as a positive control, these same membranes from follicular somatic cells were shown, as expected, to contain the somatic (130 kDa) LDL receptor. Ligand blotting was also performed on oocyte membranes with biotin-labelled TTR (Fig. VI.2). The 115 kDa TTR-binding component was again detected (along with a lower and higher molecular mass component) and could be competed by TTR (Fig. VI.2, lane 1) but not by the milk proteins present in the ligand blot buffer. Thus, by different blotting methods and with different membrane preparations, the 115 kDa species was detected using human or chicken TTR.

In order to identify the TTR receptor by an alternate method in which the membrane protein would be in the soluble phase, crosslinking reactions were performed with ^{125}I-TTR and oocyte membrane detergent extract followed by TTR-specific immunoprecipitation (Fig. VI.3). The results show the presence of a TTR-membrane protein complex of approximately 190 kDa (i.e., a tetrameric TTR receptor of ~130 kDa) and a minor component of approximately 170 kDa. Possibly, the minor component is a breakdown product of the major reactive protein; in ligand blots a TTR-binding species ~30 kDa smaller than the 115 kDa species was frequently observed. Alternatively, the 170 kDa species represents a different TTR-binding component. The major bands (13 and 33 kDa) seen in Figure VI.3, lane 1, represent the TTR monomer and dimer, respectively (188, 189). These bands are undiminished when excess unlabelled human TTR was included in the incubations (Fig. VI.3, lane 2) because the anti-

chicken TTR antibody does not recognize human TTR (cf. Figs. III. 7 and IV.2). Other minor bands seen in lanes 1 and 2 (Fig. VI.3) may represent higher order TTR structures (e.g., tetramers or minor levels of crosslinked RBP-TTR) and/or products of the oxidative action of Iodo-gen™.

Ligand blots with unsolubilized oocyte membranes did not reveal the presence of the 115 kDa or any other TTR-binding components mentioned above. Also, ligand blot analysis of serum did not show any TTR-binding components; this suggests that the 115 kDa species is not cleaved from the membrane and transported in the circulation like some other receptors, e.g., transferrin receptor (190).

B. BINDING OF THE TTR-RBP COMPLEX TO THE OOCYTIC TTR RECEPTOR

Cellular uptake of TTR from the circulatory system of vertebrates has been studied very little and the few results obtained are controversial. Many questions remain about the fate of serum TTR. Which cell types can interact with TTR? Do the ligands of TTR (i.e. thyroid hormones and retinol-RBP) play any role in its cellular interactions? Does the interaction lead to endocytosis of TTR and/or the other ligands? TTR uptake has been shown to occur in cultured human HepG2 hepatoma cells, and has been proposed to occur in other human and rat cells (e.g., adenocarcinoma, primary hepatocytes, neuroblastoma) by a receptor-mediated mechanism (144). The effects of RBP interaction on TTR uptake have not been examined to date.

Based on the data presented in Sections IV-VIA and the fact that TTR is a homotetramer, it may be possible for TTR to interact with both RBP and the oocytic TTR receptor which was identified in this study. Ligand blots of chicken oocyte membranes with chicken RBP-TTR complex followed by an anti-chicken TTR IgG suggested an interaction of the 115 kDa TTR receptor with the complex. It was not ruled out, however, that free TTR in the complex preparation was the species interacting with the receptor. Thus, uptake of the complex by this TTR receptor remains as a hypothesis to be tested.

Fig. VI.1 Ligand blotting analysis of cellular membranes for the presence of transthyretin-binding components. The blots were performed as described in Chapter II. Membrane proteins from chicken oocytes (lanes 1-3, 120 µg each), granulosa cells (lane 4, 200 µg) and embryonic fibroblasts (lane 5, 200 µg) were transferred to nitrocellulose and probed with ^{125}I-TTR in the absence (lanes 1, 4, and 5) or presence (lane 2) of a 30-fold molar excess of unlabelled TTR. Lane 3 was incubated with ^{125}I-vitellogenin (20 µg/ml; 50 cpm/ng) to reveal the two known lipoprotein receptors. M_r standards (kDa) are indicated to the left of lane 1.

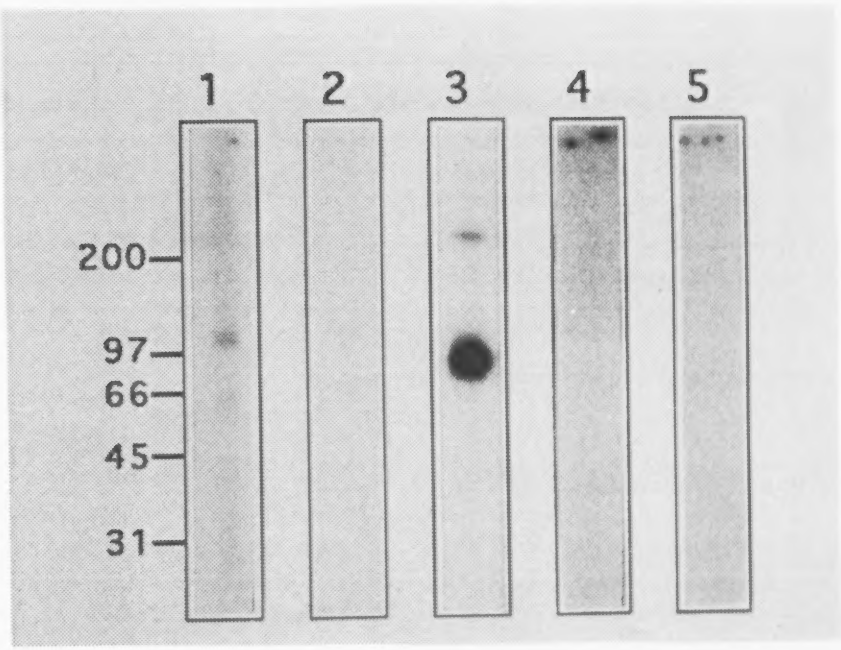

1
2
3
4
5
200
97
66
45
31

Fig. VI.2 Ligand blot analysis of chicken oocyte membranes for TTR-binding components. Biotinylated TTR was incubated with nitrocellulose strips containing immobilized oocyte membrane proteins separated by SDS-PAGE. The ligand blot was performed in the presence (lane 1) or absence (lane 3) of a 100-fold molar excess of unlabelled TTR, and binding was detected by streptavidin-HRP followed by ECL. A control strip was probed with only streptavidin-HRP (lane 2). Molecular mass standards (kDa) are indicated to the left of lane 3.

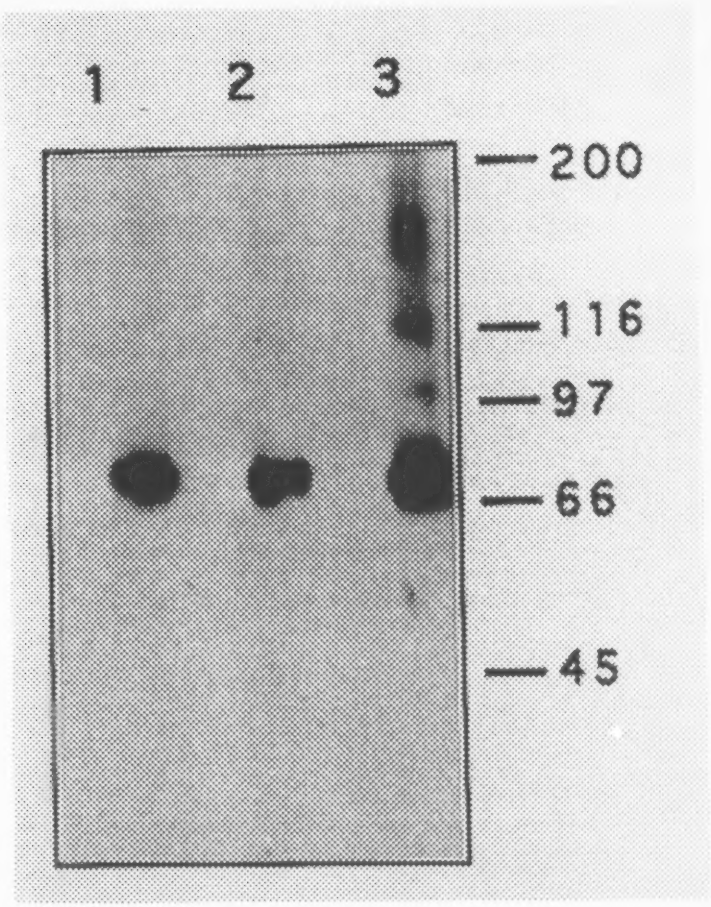
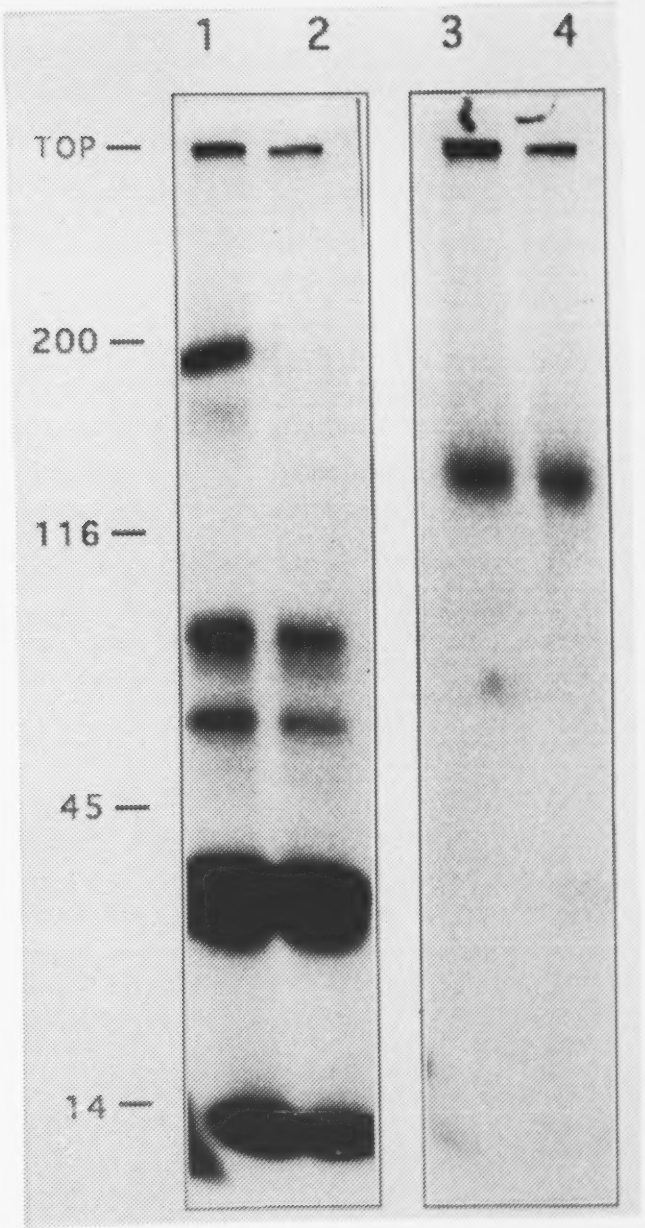

1
2
3
200
116
97
66
45

Fig. VI.3 Chemical crosslinking analysis of transthyretin (TTR)-binding components on chicken oocyte membranes. Detergent-solubilized membranes were incubated with chicken ^{125}I-TTR in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of a 100-fold excess of unlabelled human TTR, crosslinked, and immunoprecipitated with rabbit anti-chicken TTR IgG (lanes 1 and 2) or non-immune IgG (lanes 3 and 4) as described in Section II.L. M_r standards (kDa) and the top of the separating gel are indicated to the left of lane 1.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CHAPTER VII

GENERAL DISCUSSION

A. NOVEL MECHANISMS OF RETINOL UPTAKE

The studies detailed in the above chapters provide the first characterization of the molecular and transport properties of serum retinol-RBP-TTR complex in relation to avian oogenesis. The results reveal that the chicken oocyte, a cell specialized for storage, is the first cell known to contain the ternary complex. The presence of exogenously derived retinol-RBP-TTR contrasts with previous results in many extrahepatic somatic cells in which no evidence for the concomitant uptake of the RBP and TTR protein moieties with retinol was found. Independent investigations of the uptake of free RBP and free TTR by primary hepatic cells and hepatoma (HepG2) cell lines have suggested that endocytosis of these proteins may occur in the liver (Section I.C). These studies are controversial, however, and the results may have been complicated by in situ synthesis and secretion of RBP (shown in Fig. 3 of Appendix A for HepG2 cells) or TTR in the cells used for the experiments. In the present study, the possibility of significant amounts of oocytic (or follicular) synthesis of RBP, TTR, and other abundant yolk/serum components is discounted directly or indirectly by (a) analyses of ovarian follicular RNA (Chapter III), (b) by the lack of general *de novo* protein synthesis of yolk components in the follicle (13, 14 and A. Vieira, unpublished observations), and (c) by cumulative oocytic uptake and yolk deposition of RBP, TTR and certain other components such as VLDL, IgG, and riboflavin-binding protein (Chapters I, IV, V and Refs 44, 45). Furthermore, in the case of TTR, direct uptake of circulatory protein by the oocyte is shown to occur in vivo (Fig. V. 1). Taken together these data suggest that the chicken oocytes, and possibly

oocytes from other oviparous species, have a different retinol uptake mechanism relative to the majority of somatic cells. Endocytosis of the RBP-TTR complex, which is the most likely oocytic retinol uptake mechanism, has not been reported for any other cell type.

In terms of general cellular retinol uptake mechanisms, I suggest that, while in chicken oocytes and possibly hepatocytes retinol enters with its protein carrier(s) as a result of specific carrier-receptor interactions, in many somatic cells it enters through some type of hydrophobic channel in the cell membrane. Uptake through such a channel would be analogous to, and may involve a similar protein to those members of the 7-transmembrane-domain family which among other functions are involved in the uptake of certain small metabolites, e.g. opioids and arachidonate metabolites (191-194). Interestingly, one member of this family, rhodopsin, has a retinoid (retinal) in its central channel; although the retinal is covalently bound, it has been shown that a rhodopsin with a noncovalently bound retinal can still function normally (195). Perhaps rhodopsin is a prototype of the retinol membrane transporter. Alternatively or in addition, the multidrug-resistance/ABC-transporter type of cell surface proteins may be able to transport retinol. These proteins are implicated in the transport of several small ligands (e.g., steroid hormones (63, 196-198) and vinblastine (196)) including the removal of various drugs from the cells of patients undergoing chemotherapy. It has been found that leukemic patients undergoing differentiation therapy with RA eventually develop resistance (10 and refs therein) to the retinoid.

B. RECEPTOR-MEDIATED ENDOCYTOSIS AND YOLK FORMATION

Selective and accumulative uptake from the serum of nutrient-binding proteins and lipoproteins such as riboflavin-binding protein, VTG, and VLDL into growing chicken oocytes has been suggested by previous studies (24, 54-57, 179, 199). In the case of VTG and VLDL, the most abundant components of yolk, it has been shown that their internalization occurs by receptor-mediated endocytosis (53-57, 181). In Section V of this thesis, the presence of both RBP and TTR in oocytic clathrin-coated vesicles (CCVs) confirms their endocytosis and suggests that the endocytic mechanism may involve a receptor(s) on the ooctyte surface. Indeed, the chicken oocyte shows an extremely high density of membrane coated pits and CCVs (49, 53, 200, 201) which reflect a very high rate of receptor-mediated endocytosis. Such an abundance of endocytic features is not observed in the somatic cells of the chicken ovarian follicle (47, 49), and it is unlikely that these follicular cells provide any significant contribution to the bulk accumulation of nutritional yolk components in the rapid growth stage of the oocyte (diameter >2 mm). From studies of somatic cells, it is known that clathrin-coated vesicles can also be generated from the Golgi as part of the biosynthetic/exocytic pathway (37 and refs therein). In the oocyte, however, the relative contribution of such exocytic vesicles to the total CCV pool is expected to be negligible in light of the much larger contribution made by the endocytic pathway. RBP biosynthesis in the follicle, for example, is shown to be unlikely (Fig. III.5); yet the protein is present in oocytic CCVs. Thus, it is likely that receptor-mediated endocytosis occurs for many of the hepatically synthesized

serum nutrient carrier proteins and lipoproteins, and is the major mechanism involved in the final growth phase of the chicken oocyte (57).

It has previously been shown that there are two (95 kDa and 380 kDa) receptors on the chicken oocyte membrane which interact with the major yolk lipoprotein components VLDL and VTG, and possibly alpha-2-macroglobulin (54-56). To date, the 95 and 380 kDa proteins represent the only two receptors characterized in this system. In chapter VI it is shown, by chemical crosslinking and ligand blotting, that TTR interacts with a different oocyte component, i.e., a ~115 kDa membrane protein. This 115 kDa receptor was not detected in ovarian follicle somatic cells. Although TTR itself clearly interacts with this putative oocyte receptor, in the present study, it is not possible to rule out that a minor amount of co-purified RBP influenced the observed interactions of TTR with its oocytic receptor. Also, it remains to be determined if the TTR receptor which was identified in this study is involved in the uptake of TTR and TTR-RBP complexes, both of which are present in chicken serum and yolk. The data presented which show similar yolk accumulation patterns of RBP and TTR, and higher ratios of these proteins relative to albumin in yolk vs. serum are consistent with a receptor-mediated uptake of the RBP-TTR complex by the oocytes.

C. SERUM TRANSPORT OF RETINOIDS AND THYROID HORMONES

The TTR-RBP complex is involved not only in the transport of retinol but also of thyroid hormones. The thyroid hormones interact

directly with the TTR component of the complex. Both the thyroid hormone, T_3, and metabolites of retinol, acting through their respective nuclear binding proteins, are known to be important regulators of embryonic development and normal adult tissues in many vertebrates including the chicken (Chapter I). The contribution of RBP and the RBP-TTR complex to the total transport of retinoids and thyroid hormones in animals such as the chicken must be placed in the perspective of other serum transporters for these substances.

In the case of serum retinoid transport, two other circulatory lipid transport systems should be considered: (i) albumin and (ii) lipoproteins. For the chicken oogenesis model presented in this thesis, the role of albumin in retinoid transport has not been defined. Although it is likely that the albumin present in the oocyte enters by a nonspecific, fluid phase mechanism and is not concentrated in the yolk relative to RBP or TTR (Fig. V.2 and 5), it is not known how much (if any) of that yolk albumin contains bound retinoic acid and whether that retinoic acid can be used in the retinoid-dependent stages of early development. It is known that circulatory RA (bound to albumin) does not compete with retinol for interaction with the RBP-TTR transport complex (85). (ii) Relative to RBP, the role of lipoproteins in retinoid or retinoid precursor delivery to the chicken oocyte is not expected to be significant under normal dietary loads of retinol or pro-retinol carotenoids (79), especially due to the protomicron lipoprotein delivery system (Sections I.A and C). The chicken oocyte is known to accumulate some types of carotenoids (15). Most of these are "unwanted" carotenoids which are not readily metabolized in the gut and liver (15). Very little accumulation of pro-vitamin A carotenoids (i.e., alpha-, beta-, and gamma- carotene) has been found in the yolk (15); it is

unlikely that they escape the breakdown in the intestine and liver. Even with low quantities of pro vitamin A carotenoids in the yolk, it is not known if the carotene-to-retinoid conversion enzymes are present in the yolk and are sufficiently active to meet the needs for the necessary retinoids (e.g., RA) at the appropriated times during the early stages of development. The same arguments may be applied to transport of lipoprotein-bound retinyl ester into the yolk. In addition, neither the plasma levels of retinyl esters nor of carotenoids are tightly regulated (Section I.C), but those of RBP are maintained within a narrow range (~2 uM in mammals (69, 87, 129, 140)). In light of the necessary control of cellular retinol levels and metabolism, this difference suggests that retinol-RBP is the important transport species in most animals.

Recent experiments involving transgenic mice with a disrupted TTR gene that did not produce any detectable circulatory TTR, confirmed the importance of RBP in retinol plasma transport and revealed that the complexation of RBP with TTR is an important factor in maintaining plasma RBP levels (188). In the transgenics, plasma retinol and RBP levels were decreased by the same extent and to very low levels (188). A similar proportional decrease of RBP and retinol serum levels has been reported in rats treated with fenretinide, a synthetic retinoid that interacts with RBP and inhibits the RBP-TTR interaction (184). In the TTR^- mice, the thyroid hormone levels were also decreased (~2-3-fold) (188). But, apparently, these low levels of retinol and thyroid hormones were sufficient to maintain a normal phenotype.

Thyroid hormones are transported by several proteins in the circulatory systems of vertebrates: albumin, TTR, thyroxine-binding globulin (TBG), and alpha-globulin (172). Birds lack a specific TBG and it

has been estimated that ~70% of non-albumin bound (>50% of total (140, 188)) thyroid hormones are carried by TTR (172). In the chicken, thyroid hormones have been reported to accumulate in the yolk during oogenesis (202). Although the role of TTR was not examined in that study, it was noted that the highest accumulation of the hormones per unit mass occurs in the smallest oocytes (202). It was shown in Section V that TTR levels relative to total yolk protein are also highest in the smaller oocytes. This supports previous suggestions that TTR is a more important carrier of THs into the oocyte (Ref. 202 and Section V.B) than the nonspecific carriers such as albumin.

The thyroid hormone, T_3, as well as various derivatives of retinol (e.g., all-trans retinoic acid and 9-cis retinoic acid) affect transcription of important regulatory genes by acting through their respective nuclear receptor proteins, as explained in chapter I. Furthermore, the nuclear receptors for T_3 and retinoids can affect each others' activities through their common heterodimerization partner, RXR. In this respect, it is interesting to note that the uptake of two important regulatory components, retinol and T_3, into the oocyte may be co-regulated as a result of their common interaction with TTR and the presence of an oocytic TTR receptor.

D. FUTURE PROSPECTS

Future studies aimed at analyzing oocytic retinol-RBP and/or TTR uptake should involve further characterization of the TTR

receptor that was identified in this study: (i) confirm the presence of the receptor in oocytic clathrin-coated pits and vesicles, (ii) clone and sequence the TTR receptor as a first step to a structural analysis and comparison of with other receptor systems, (iii) confirm that the TTR receptor can bind and transport the RBP-TTR complex.

Other future prospects of interest which are based directly on the findings reported in this thesis include: (i) use of the chicken RBP gene as a tool to uncover possible mechanisms of estrogen-mediated transcriptional repression, (ii) analysis of the role of putative 3' UTR regulatory sequences in RBP mRNA stability and translatability, (iii) use of RBP, TTR, apo D, apo AI as markers for studying endocytosis and yolk deposition pathways of the oocyte, (iv) a study of the mobilization of retinol and its transport complex from storage sites in the yolk to the embryo.

APPENDIX A

CLASSES OF LAYING HEN SERUM YOLK PRECURSORS BASED ON THEIR HEPATIC ESTROGEN RESPONSE AND BIOCHEMICAL FUNCTIONS

1. Estrogen Effects on Avian Serum TTR and Vitellogenin Levels

At the onset of egg-laying, the sudden rise in circulatory estrogen levels causes a dramatic induction of hepatic synthesis (Section III.B) and, subsequently, the serum levels of egg yolk precursor proteins and lipoproteins (18, 19, 21-23, 26). The effect of estrogen is readily observed and typically studied in roosters by injecting them with 17ß-estradiol (18, 19, 21-23, 26). Previously characterized proteins and lipoproteins, e.g. vitellogenin, very low density lipoprotein (VLDL), and riboflavin-binding protein which are induced in the hen, taken up by the oocyte, and deposited in the yolk, are also induced in the rooster model (18, 19, 21-23, 26). However, estrogen treatment does not significantly change serum TTR levels relative to those in control roosters over a period of at least 125 hr (Fig. 1, upper panel). As expected, serum vitellogenin is strongly induced, with peak values reached approximately 60 hr following hormone administration (Fig. 1, lower panel). In addition, TTR levels in laying hens are not higher than in roosters (Fig. 2). Although the above experiments do not rule out that estrogen affects both the rate of synthesis and catabolism (or clearance) of serum TTR, it can be concluded that TTR belongs to a class of proteins whose serum levels are not significantly changed by estrogen, but which are nevertheless efficiently transported to the oocyte. One possible explanation for this difference between TTR and

most major yolk proteins is that large fluctuations in the levels of TTR could adversely affect the controlled delivery of important regulatory ligands, thyroid hormones and retinol, to peripheral cells. Consistent with this idea, the results below suggest that serum levels of RBP are similarly not significantly influenced by estrogen.

2. Effects of Acute and Repeated Estrogen Treatments on serum RBP Levels

It has previously been shown that estrogen treatment of roosters leads to an acute decrease in RBP hepatic RNA levels (Chapter III.A). But estrogen does not appear to change the production of RBP from a human liver-derived (HepG2) cell line (Fig. 3). In the estrogen-treated rooster model, Fig. 4A (upper panel) shows that RBP serum levels are not changed significantly by estrogen, unlike those of VTG (lower panel). In addition, a repeated estrogen treatment performed 11 days after the first did not significantly raise or decrease RBP levels (Fig. 4B, upper panel); but this second treatment again increased VTG levels dramatically (lower panel). No difference in the RBP serum levels of the laying hen was noticed, relative to rooster (Fig. 2); it is known that VTG levels in the laying hen are very high while in the normal rooster they are undetectable (18-23).

3. Overview of Estrogen Effects and the Establishment of Different Classes of Laying Hen Serum Yolk Precursors

As shown above, VTG serum levels increase dramatically in response to estrogen treatment of roosters (and are known to be highly

elevated in laying hens (18-23)). Chicken serum levels of VLDL, the other major circulatory yolk precursor, are also greatly increased by estrogen (17-23). The data presented in this appendix suggest that the circulatory steady state levels of yolk precursors in the laying hen, determined mainly by the balance between hepatic synthesis/secretion and oocyte uptake, appear to fall into two categories: (i) high serum levels of some components such as lipoproteins, which contribute most to yolk *mass*, and some vitamin-binding proteins, e.g. riboflavin- and biotin-binding protein (24, 179); and (ii) lower serum levels of others, e.g. RBP and TTR. It appears that estrogen responsiveness at the level of synthesis is one of the key features responsible for the existence of these two categories. Interestingly, the vitamin-binding protein members of group (i) carry enzyme cofactors or their precursors and are also synthesized in the oviduct under the influence of estrogen (e.g., riboflavin-, biotin-, thiamin-binding proteins), while members of group (ii) carry direct effectors of gene regulation or their precursors, e.g., RBP, TTR, vitamin D-binding protein (and possibly apo D, see Appendix B).

Fig. A1. Effects of estrogen treatment on serum transthyretin (TTR) and vitellogenin (VTG) levels in roosters. Each lane of the Western blots contains the proteins of 2 µl rooster serum treated with 50 mM DTT. Polyclonal antibodies against TTR and VTG were used to detect the two respective proteins at the following times post-estrogen treatment: lane 1, 12 h; lane 2, 26 h; lane 3, 33 h; lane 4, 57 h; lane 5, 81 h; lane 6, 125 h. Lane 7 represents the untreated control. M_r standards are indicated to the left of the respective panels.

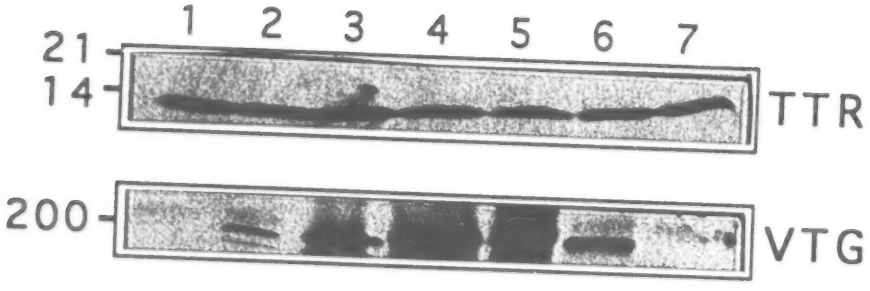

1 2 3 4 5 6 7
21
14
TTR
200
VTG

Fig. A2. Comparison of serum TTR and RBP levels in the laying hen (lanes 2) and rooster (lanes 1) serum. Each lane contains 2 µl of serum. The samples were heated (95°C, 5-10 min) under reducing conditions, subjected to SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed on the serum proteins with polyclonal antibodies against TTR (A) or RBP (B).

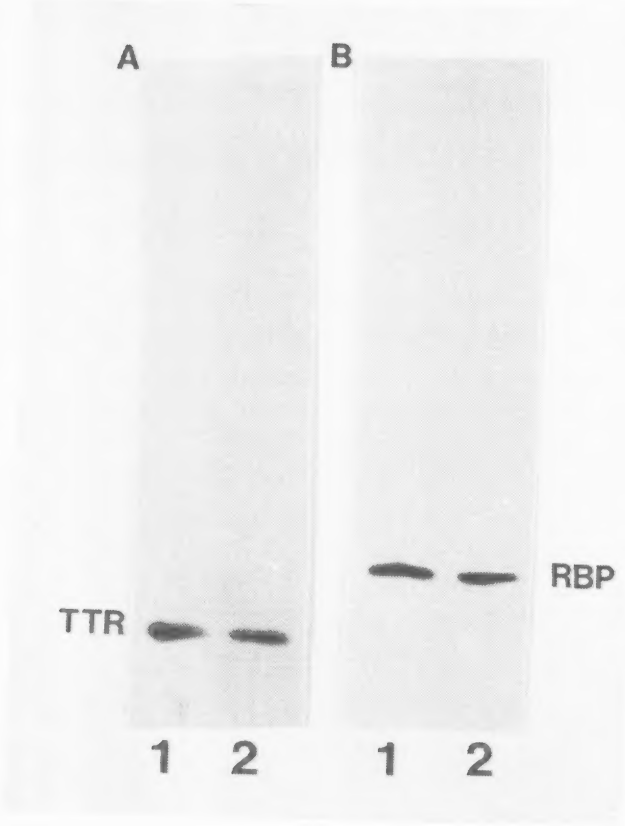

A
B
TTR
RBP
1
2
1
2

Fig. A3. Analysis for secretion of RBP and TTR by a human hepatocyte-derived cell line (HepG2). The cells were cultured in serum-free Weymouth's media as described in Materials and Methods, and devided into 3 groups of 20 dishes each. When half-confluency was reached, the cells were treated with 20 mM (lanes 2 and 6) or 100 mM (lanes 1 and 5) estrogen, or the equivalent volume (200µl) of organic carrier, propylene glycol (lanes 4 and 8). The media was concentrated 3-fold and 40 µl aliquot was subjected to Western blotting analysis for the presence of TTR (lanes 1-4) or RBP (lanes 5-8). Lanes 3 and 7 do not contain any sample. M_r standards are indicated to the left of lane 1 (from top to bottom): 200 kDa, 69 kDa, 29 kDa, 18 kDa, 14 kDa.

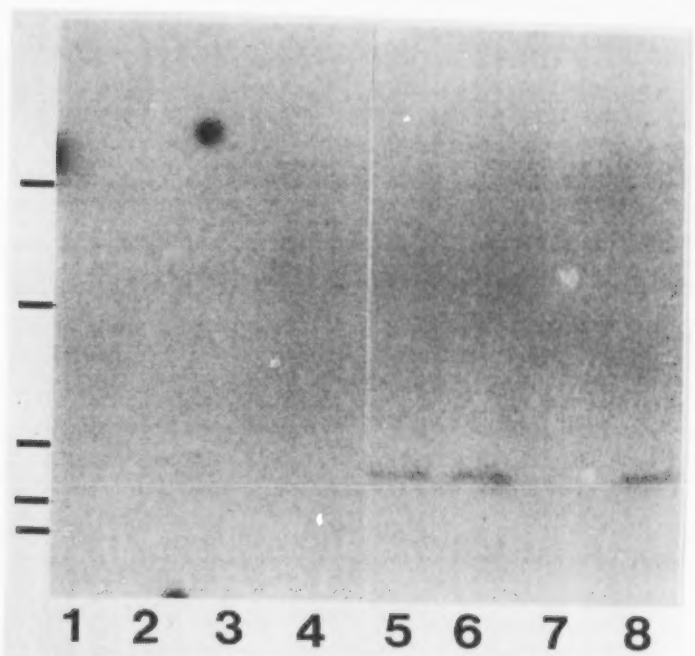
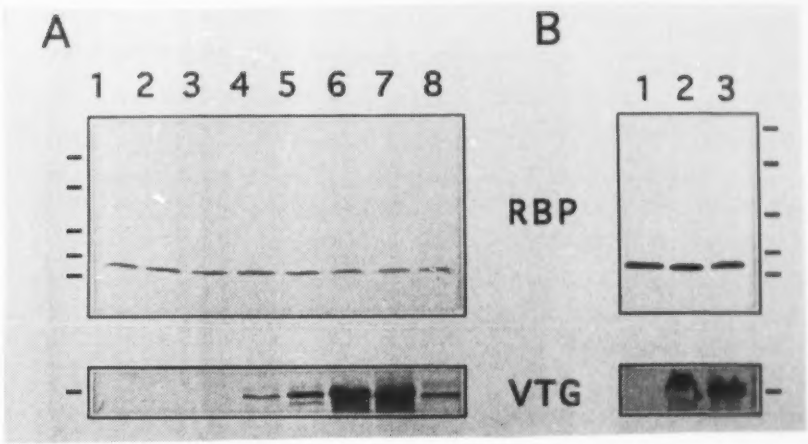

1 2 3 4 5 6 7 8

Fig. A4. Effects of estrogen treatment on rooster serum retinol-binding protein (RBP) and vitellogenin (VTG) levels. Each lane of the Western blots contains the equivalent of 2 µl of rooster serum treated with 50 mM DTT. (**A**) Polyclonal antibodies against RBP and VTG were used to detect the two respective proteins at the following times post-estrogenization: lane 1, 0 hr (control); lane 2, 4 hr; lane 3, 12 hr; lane 4, 26 hr; lane 5, 33 hr; lane 6, 57 hr; lane 7, 81 hr; lane 8, 125 hr. Molecular mass standards are indicated by the bars to the left of the top panel (21 kDa, 31 kDa, 45 kDa, 66 kDa, 97 kDa) and bottom panel (200 kDa). (**B**) The same antibodies were used to detect RBP and VTG in rooster serum either before estrogen treatment (control, lane 1) or 24 hr (lane 2) and 49 hr (lane 3) after a secondary estrogen treatment performed 11 days after the first. Molecular mass standards are indicated by the bars to the right of the top panel (21 kDa, 31 kDa, 45 kDa, 66 kDa, 97 kDa) and bottom panel (200 kDa).


A
B
1 2 3 4 5 6 7 8
1 2 3
RBP
VTG

APPENDIX B

CHICKEN APOLIPOPROTEIN D AND HDL

During the course of the studies on retinol transport to the oocyte, I initiated a comparative study on an RBP homologue (i.e., a lipocalin) whose physiogical role was poorly understood, apolipo-protein (apo) D. A few selected apects of this study are presented in this appendix as a complement for the RBP and TTR endocytosis data discussed in Section V.

Apo D is an unusual apolipoprotein with respect to structure and sites of synthesis; and although its in vivo ligand has not yet been identified, it can bind bilirubin, progesterone, and other steroids in vitro (203). In the circulatory system of certain mammals, apo D has been detected in association with high density lipoproteins (203-205). I used an established monoclonal anti-human apo D antibody which recognizes a non-carbohydrate epitope of the denatured, reduced protein (205, 206) to show the presence of apo D in the first non-mammalian species, the chicken. The avian apo D has the same molecular mass (29 kDa) as the human protein and associates with the plasma HDL fraction (Fig. 1A). Very little apo D was detected on chicken VLDL or LDL. In addition to the 29 kDa apo D species, an immunoreactive 24 kDa protein in chicken serum and purified HDL (Fig. 1A) was also detected. The nature of this 24 kDa species remains unknown; such a species has not been observed in any of the mammalian systems examined so far. Estrogen treatment of roosters did not significantly change the serum levels of either the 29 kDa or the 24 kDa species.

The chicken 29 kDA apo D (and the 24 kDa immunoreactive species) was also present in the yolk of the rapidly growing oocyte (Fig.

1B). And clathrin-coated vesicles from chicken oocytes contain apo D (and the 24 kDa species) (Fig. 2, lane 2) as well as apo AI (Fig. 2, lanes 3 and 4), the major protein moiety of HDL. These data are compatible with oocytic endocytosis of serum apo D and AI, possibly in association with the HDL particle (P.M. Vieira et al., manuscript submitted).

Utilization of yolk during embryonic development involves the transport and metabolism of large quantities of lipid within the egg. It is conceivable that this is the function of apo D (and of other apolipoproteins, cf. Ref. 57) in the yolk. Indeed, the stored apo D (and the 24 kDa species) in the yolk does not appear to be significantly degraded, and should be competent to carry out any subsequent lipid transport functions. In other physiological situations where mobilization of large quantities of lipids is crucial, high levels of apo D expression have been related to lipid transport, for example, in myelin reconstruction during nerve regeneration (205-208) and during erythrocyte degradation in the spleen (205). In addition, considering the lipocalin structure of apo D and its ability to bind certain steroid hormones, it is likely that it transports important lipophilic ligand(s) into the oocyte. The accessibility of the chick embryo and of yolk apo D may help to delineate the exact function(s) of this unusual apolipoprotein.

Fig. B1. Presence of apo D on chicken serum high-density lipoproteins (HDL) and in oocytic yolk. The proteins in each lane were subjected to SDS-PAGE under reducing conditions and transferred to nitrocellulose. **(A)** Chicken HDL (30 μg of protein) is probed with a mouse monoclonal anti-apo D IgG (lane 1) or with rabbit anti-apo AI polyclonal IgG (lane 3) followed by either rabbit anti-mouse IgG and ^{125}I-protein A (lane 1) or ^{125}I-protein A only (lane 3). As a control (lane 2), the same HDL sample is probed with rabbit anti-mouse IgG and ^{125}I-protein A. **(B)** Oocytic yolk proteins (80 μg), were probed for apo D as (A). M_r standards (in kDa) are indicated at the left.

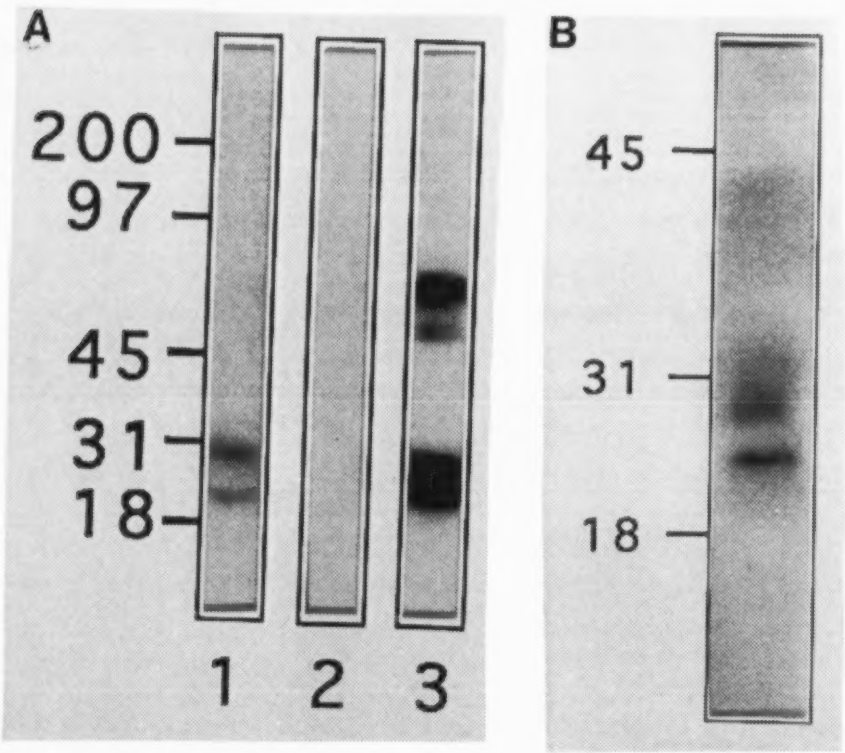
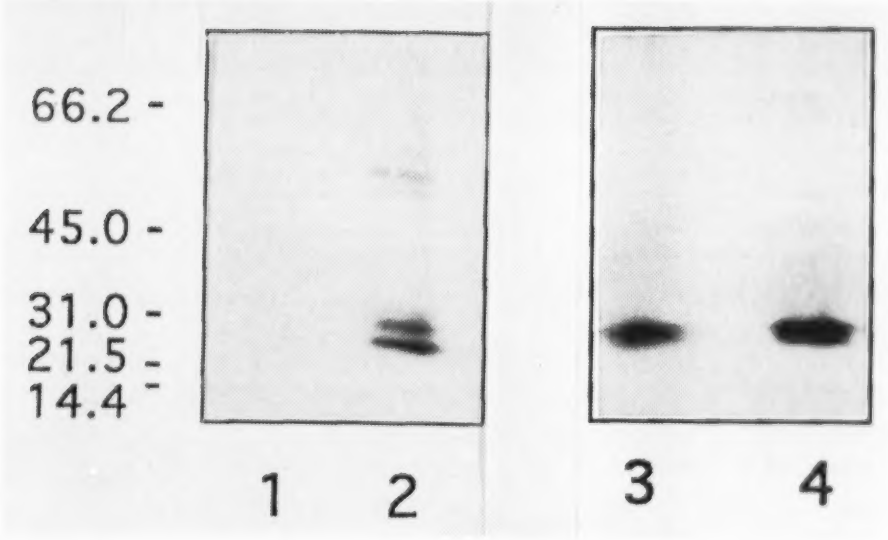

A
200
97
45
31
18
1
2
3
B
45
31
18

Fig. B2. Presence of apo D and apo AI in chicken oocyte clathrin-coated vesicles. Western blots of the coated vesicle contents for apo D (lanes 1 and 2) and apo AI (lanes 3 and 4), were performed as described in the legend to Fig. 1 except that protein A-HRP and ECL detection were used; the samples were either reduced with 50 mM dithiothreitol (lanes 2 and 4) or non-reduced (lanes 1 and 2). It is known that the anti-apo D mAb used is specific for the reduced, denatured form of the protein (see text). M_r standards (kDa) are shown to the left of lane 1. Apo AI migrates between the two putative apo D species.


66.2
45.0
31.0
21.5
14.4
1
2
3
4

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